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(54) 【発明の名称】 ハイブリッド抗原タンパク質、それを発現する組み換えウイルス、及びその製造方法

(57) 【要約】

【目的】 ワクチンや診断薬として有用なウイルスの構成タンパク質と病原ウイルスの小型ペプチドエピトープとを有するハイブリッド抗原タンパク質を開発する。

【構成】 ウイルスの構成タンパク質遺伝子と病原ウイルスの小型ペプチドエピトープ遺伝子とを有する組み換えウイルスを作製し、培養細胞内でこの組み換えウイルスを発現させることにより、ウイルスの構成タンパク質と病原ウイルスの小型ペプチドエピトープとを有するハイブリッド抗原タンパク質を製造する。

【特許請求の範囲】

【請求項1】 ウイルスの構造タンパク質と構成アミノ酸が300以下である病原ウイルスのペプチドエpitepとを有するハイブリッド抗原タンパク質。

【請求項2】 ウイルスの構造タンパク質をコードする遺伝子と構成アミノ酸が300以下である病原ウイルスのペプチドエpitepをコードする遺伝子とをウイルスの増殖に非必須な領域に組み込んだ組み換えウイルス。

【請求項3】 請求項2記載の組み換えウイルスを用いた請求項1記載のタンパク質の製造方法。

【発明の詳細な説明】

【0001】

【産業上の利用分野】 本発明は、ウイルスの構造タンパク質と構成アミノ酸が300以下である病原ウイルスのペプチドエpitepとを有するハイブリッド抗原タンパク質、それを発現する組み換えウイルス、それを用いたハイブリッド抗原タンパク質の製造方法に関する。

【0002】

【従来の技術】 近年、種々の病原ウイルス、細菌、原虫などの抗原タンパク質において、感染防御に重要な部分のペプチドエpitepが見いだされており、ワクチンとして利用することが試みられている(R. Arnon and M. Shapira, Modern Approaches to Vaccines, Molecular and Chemical Basis of Virus and Immunogenicity (R. M. Chenock et al. eds.) p. 109, Cold Spring Harbor (1984))。しかしながら、これらの比較的短鎖のペプチドワクチンは一般的に抗原性が低いため、キーホールリンペットヘモシアニンなどと結合させた上に、アジュバントと組み合わせ使用するものであり、アジュバントによる炎症などの副作用が起こるという欠点があった。

【0003】 一方、組み換えウイルスによる生ワクチンは安全性も高く、優れた抗原性を示すことが報告されている(特開昭64-74982号公報など)。そこで、組み換えウイルスにより合成ペプチドワクチンに相当するペプチドエpitepを効率よく発現させることができれば、上記ペプチドワクチンの抗原性が低いという欠点が改善できると考えられる。

【0004】 しかし、本発明者らの実験によると小型のペプチドエpitepを組み換えウイルスを用いて発現するために、従来通りの大型の抗原タンパク質を発現する方法(特開昭64-74982号公報など)をそのまま用いても、小型ペプチドの細胞内での安定性などに問題があり、十分な発現量を得ることはできなかった。

【0005】

【発明が解決しようとする課題】 そこで本発明者らは、かかる従来技術の下で、比較的短鎖のペプチドエpitep

を発現させるべく鋭意検討を進めた結果、ウイルスの構造タンパク質をコードする遺伝子と病原ウイルスのペプチドエpitepをコードする遺伝子とを組み込んだ組み換えウイルスを用いると、効率よくウイルスの構造タンパク質と小型エpitepとを有するハイブリッド抗原タンパク質が得られることを見だし、本発明を完成するに至った。

【0006】

【課題を解決するための手段】 かくして本発明によれば、第1の発明として、ウイルスの構造タンパク質(以下、構造タンパク質という)と構成アミノ酸が300以下、好ましくは、構成アミノ酸が10~150、より好ましくは15~100、さらに好ましくは20~50である病原ウイルスのペプチドエpitep(以下、小型エpitepという)とを有するハイブリッド抗原タンパク質(以下、ハイブリッドタンパク質という)が提供される。

【0007】 また、第2の発明としてそのハイブリッドタンパク質を発現するウイルスの構造タンパク質をコードする遺伝子(以下、構造タンパク質遺伝子という)と構成アミノ酸が300以下である病原ウイルスのペプチドエpitepをコードする遺伝子(以下、小型エpitep遺伝子という)とをウイルスの増殖に非必須な領域に組み込んだ組み換えウイルスが提供される。さらに、第3の発明として、この組み換えウイルスを用いたハイブリッドタンパク質の製造方法が提供される。

【0008】 本発明において組み換えウイルスの作製に供されるウイルス(以下、宿主ウイルスという)は、一般の遺伝子組み換え技術に使用されるウイルスを使用すればよく、例えば、ワクチニアウイルス、バキュロウイルス、アビボックスウイルスなどが挙げられる。ペプチドエpitepの由来となった病原ウイルスが感染する動物と同じ種に感染するウイルスを使用することが好ましく、例えば、ヒト免疫不全ウイルス(以下、HIVという)由来のペプチドエpitepを組み込むのであれば、ヒトに感染するワクチニアウイルスを使用すればよい。

【0009】 ワクチニアウイルスの例としては、WR株(J. Virol., 49, 857, (1984))、リスター株、温度感受性リスター株(特開昭62-44178号)、New York Board of Health株、LC16m8株などの種痘ワクチン株が例示され、バキュロウイルスの例としては、オートグラフ・カリフォルニカ(Autographa californica)、トリコプルシア・ニ(Trichoplusia ni)、ラキプルシア・オウ(Rachiplusia ou)、ガレリア・メロネラ(Galleria mellonella)、ボンビクス・モリ(Bombyx mori)などが挙げられ、アビボックスウイルスの例としては、ATCC VR-251、ATCC VR-250、ATCC VR-22

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9、ATCC VR-249、ATCC VR-228、西ヶ原株、NP株（鶏胎化鳩痘毒中野系株）などが挙げられる。

【0010】本発明において使用される構造タンパク質遺伝子は、ウイルスの構造タンパク質として機能するものであれば特に限定されないが、例えば、ワクチニアウイルス主要外膜抗原p37（J. Virol., 39, 903 (1981)）、ワクチニアウイルスヘマグルチニン（Virology, 150, 451 (1986)）、ワクチニアウイルスAg35抗原（J. Virol., 181, 671 (1991)）などが挙げられる。また、これらのタンパク質遺伝子は、構造タンパク質として機能するものを発現する限りにおいて修飾されたもの（アミノ酸の欠損、増加、変更を含む）であってもよい。

【0011】このようなタンパク質遺伝子のうち、免疫原性の点から考えると、ウイルスの外膜タンパク質が好ましく、ウイルスの外膜タンパク質であって感染細胞の表面に発現するものがより好ましい。このような遺伝子の具体例としては、ワクチニアウイルス主要外膜タンパク質p37（J. Virol., 58, 757 (1986)）、ワクチニアウイルスヘマグルチニンなどが挙げられる。また、組み込むウイルスと同じ属に属するウイルスの構造タンパク質を使用する方が好ましい。

【0012】組み込む構造タンパク質遺伝子の大きさは、構造タンパク質の由来となるウイルスが組み込むウイルス（以下、宿主ウイルスという）と同じ種のウイルスであるならば、宿主ウイルスの増殖に影響はないので特に限定されないが、宿主ウイルスと異種のウイルス由来の構造タンパク質遺伝子を使用する場合、宿主ウイルスが増殖できる程度の大きさでなければならず、余り大きな遺伝子を組み込むことはできない。このような場合の遺伝子の大きさは、通常、10,000塩基以下、好ましくは2,000塩基以下である。

【0013】本発明において使用される小型エпитープをコードする遺伝子は、病原ウイルスのエпитープであって構成アミノ酸が300以下、好ましくは10~150、より好ましくは15~100、さらに好ましくは20~50であるものをコードする遺伝子であり、例えば、HIVのHGP30をコードする遺伝子（Proc. Natl. Acad. Sci. USA, 84, 2951-2955 (1988)）や、HIVのV3をコードする遺伝子（Proc. Natl. Acad. Sci. USA, 85, 1932 (1988)）などが挙げられる。

【0014】また、これらのタンパク質遺伝子は、小型エпитープとして機能するものを発現する限りにおいて修飾されたもの（アミノ酸の欠損、増加、変更を含む）であってもよく、さらに天然のウイルスから得られたものであっても、ウイルス遺伝子のcDNAの一部であつ

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ても、また合成されたものであってもよい。

【0015】本発明のハイブリッドタンパク質は、前述の宿主ウイルスに上記の構成タンパク質遺伝子と小型エпитープ遺伝子とを組み込んだ組み換えウイルスを作製し、これを適当な宿主細胞内で発現させることにより製造される。このようなハイブリッドタンパク質の具体例としては、ワクチニアウイルス由来p37タンパク質とHIV由来HGP30タンパク質とのハイブリッドタンパク質、ワクチニアウイルス由来p37タンパク質とHIV由来V3タンパク質とのハイブリッドタンパク質などが挙げられる。

【0016】以下に、本発明の組み換えウイルスおよびハイブリッドタンパク質の一般的な作製方法について説明する。

【0017】（第1の組み換えベクターの作製）第1の組み換えベクターは、目的とするハイブリッドタンパク質を作製するために使用する構成タンパク質遺伝子を含んでいる。宿主ウイルスに構成タンパク質遺伝子と小型エпитープ遺伝子とを相同組み換えにより挿入する場合、宿主ウイルスの増殖に非必須な遺伝子の一部が構造タンパク質遺伝子の両端にある必要がある。従って、組み込むウイルスが宿主ウイルスと異なる種のウイルスの場合は、宿主となるウイルスの増殖に非必須な遺伝子領域の一部を構成タンパク質遺伝子の両端に存在させる必要がある。

【0018】第1の組み換えベクターの作製の材料となるベクターは、特に限定されないが、例えばpBR322、pBR325、pUC18などのごときプラスミドや、λファージ、M13ファージなどのごときファージ、pHC79（Gene, 11, 291, (1980)）などのごときコスミドが例示される。

【0019】第1の組み換えベクターの作製は、常法に従って行うことができる（特開昭62-44178号公報、特開平1-285198号公報、特開平1-168279号公報など）。例えば構成タンパク質としてワクチニアウイルス由来のもの、宿主ウイルスとしてワクチニアウイルスを用いる場合、J. Virol. Methods, 2, 175-179 (1981) 記載の方法にしたがって調製したワクチニアウイルス由来のDNAを使用するワクチニアウイルス構成タンパク質遺伝子の一部、およびその周辺部を適当な制限酵素で切り出し、適当なベクターに組み込めばよい。

【0020】（第2の組み換えベクターの作製）このようにして作製した第1の組み換えベクター内の構造タンパク質遺伝子のいずれかの部分に目的とする小型エпитープ遺伝子を挿入または付加して第2の組み換えベクターを作製する。挿入、または付加にあたっては構造タンパク質遺伝子と小型エпитープ遺伝子が両方とも正しいアミノ酸翻訳の読み枠でつながれ、プロモーター部分もふくめて両方の遺伝子が共に破壊されないように設計さ

れねばならない。

【0021】構造タンパク質遺伝子の開始コドンのすぐ上流へ小型エビトープ遺伝子を付加する場合は、小型エビトープ遺伝子の最上流に開始コドンが必要であり、構造タンパク質遺伝子の終止コドンのすぐ下流へ小型エビトープ遺伝子を付加する場合は、構造タンパク質遺伝子の終止コドンの除去と小型エビトープ遺伝子の最下流にストップコドンを作る必要がある。

【0022】(第3の組み換えベクターの作製) 第2の組み換えベクターに対し、組み換えウイルスの選択を容易にするためのマーカー遺伝子が常法により挿入され、第3の組み換えベクターが作製される。

【0023】マーカー遺伝子は、特に限定されないが、例えば β -ガラクトシダーゼ遺伝子(Molecular And Cellular Biology, 5, 3403 (1985))、Ecogpt遺伝子(J. Virol., 62, 1849-1854 (1988))などが例示される。マーカー遺伝子は、プロモーターの下流につないだ状態で使用される。

【0024】マーカー遺伝子につなぐプロモーターとしては、組み込むウイルス内で機能するものであれば特に限定されず、例えば、ワクチニアウイルス内で発現するものとしては、7.5Kポリペプチドをコードするワクチニアウイルス遺伝子のプロモーター(以下、7.5Kプロモーターという)、19Kポリペプチドをコードするワクチニアウイルス遺伝子のプロモーター(以下、19Kプロモーターという)、11Kポリペプチドをコードするワクチニアウイルス遺伝子のプロモーターなどが例示され、バキュロウイルス内で発現するものとしては、バキュロウイルスのポリヘドリンをコードするバキュロウイルス遺伝子のプロモーター、バキュロウイルスの10Kポリペプチドをコードするバキュロウイルス遺伝子のプロモーターなどが例示され、アピボックスウイルス内で発現するものとしては、アピボックスウイルスのチミジンキナーゼをコードするアピボックスウイルス遺伝子のプロモーター、7.5Kプロモーター、19Kプロモーターなどが例示される。

【0025】プロモーターの下流につないだマーカー遺伝子は、第2の小型エビトープ遺伝子が挿入、または付加されたワクチニアウイルス構成タンパク質遺伝子の近傍で挿入されて、第3の組み換えベクターが作製される。これら第1、第2および第3の組み換えベクターの構築当たっては、遺伝子操作の容易な大腸菌の系を用いればよい。

【0026】(組み換えウイルスの構築とタンパク質の製造) 前述の方法により得た第3の組み換えベクターを予めウイルスに感染させた細胞に移入し、ベクターDNAとウイルスゲノムの遺伝子間に相同組み換えを起こさせ、組み換えウイルスを構築する。組み換えウイルスの構築に当たっては、常法に従って行えばよく、例えば、

リン酸カルシウム共沈法、リボソーム法、マイクロインジェクション法、エレクトロポレーション法などによって第3の組み換えベクターをウイルス内に移入させ、得られる組み換えウイルスを含むウイルス集団を適当な培地上に培養する。

【0027】第3の組み換えベクターで挿入されたマーカー遺伝子に適した方法でブランクを形成させ、目的の組み換えワクチニアウイルスの候補株を得る。マーカー遺伝子として、 β -ガラクトシダーゼ遺伝子を用いた場合には、ブランクを形成させた後、ハロゲン化インドリル β -D-ガラクトシダーゼ(以下、ブルオギャルという)および寒天を含有したイーグルMEMを重層し、一晩後に青色に染色されるブランクを目的とする組み換えワクチニアウイルスの候補株とすればよい。

【0028】これらの候補株の中から目的とする組み換えウイルスを選択する方法は、目的とする小型エビトープ遺伝子をプローブとするハイブリダイゼーション法を利用してブランクを純化するか、あるいは目的とする小型エビトープに対する抗血清またはモノクローナル抗体を用いるイノムアッセイをすればよい。このようにして得られた組み換えウイルスを宿主細胞に感染させて、培養するとハイブリッドタンパク質が産生される。

【0029】ここで用いられる細胞は、宿主となるウイルスが感染するものであれば特に限定されない。宿主となるウイルスがワクチニアウイルスである場合、例えば、TK-143(ヒト骨肉腫由来)、FL(ヒト羊膜由来)、Hela(ヒト子宮頸部癌由来)、KB(ヒト鼻咽癌由来)、CV-1(サル腎由来)、BSC-1(サル腎由来)、RK13(ウサギ腎由来)、L929(マウス結合組織由来)、CE(鶏胚)、CEF(鶏胎児線維芽細胞)などが例示され、バキュロウイルスを使用する場合、例えば、スポドプテラ・フルギペルダ(*Spodoptera frugiperda*)由来のSf9細胞などが例示され、アピボックスウイルスを使用する場合、例えば、CE(鶏胚)、CEF(鶏胎児線維芽細胞)などが例示される。

【0030】このようにして産生されたハイブリッドタンパク質は宿主細胞が活着している間は通常宿主細胞内に大量に存在するので、細胞を集め破碎して回収すればよい。ハイブリッドタンパク質は公知の常法、例えば塩析、ゲル濾過、イオン交換及びアフィニティークラムクロマトグラフィーによる分離法、高速液体クロマトグラフィー、電気泳動による分画法等を適宜組み合わせる事により精製できる。

【0031】

【発明の効果】かくして本発明によれば、小型エビトープを構造タンパク質とのハイブリッド抗原タンパク質として発現することができ、このタンパク質はコンポーネントワクチンの抗原として利用でき、また、組み換えウイルスは、生ワクチンとしての利用が期待される。

【0032】

【実施例】以下に実施例をあげて本発明をさらに具体的に説明する。

(実施例1) ワクチニアウイルス主要外膜抗原p37遺伝子およびその周辺のクローニングによる第1の組み換えプラスミドpHP37の作製(図1参照) ワクチニアウイルスゲノムDNAの調製は、まず、ワクチニアウイルスLC16mO株(臨床とウイルス、3(3)13-19(1975)、以下、ワクチニアウイルスmO株という)を常法により培養し、J. Virol. Methods、2、175-179(1981)記載の方法により行った。

【0033】このゲノムDNAを制限酵素PstIと制限酵素HindIIIで切断し、p37遺伝子を含む4452bpのDNA断片を回収した。pUC18を制限酵素PstIと制限酵素HindIIIで切断し、先に得たDNA断片を挿入し、第一の組み換えプラスミドpHP37を作製した。pHP37の確認は、Virology、179、247-266(1990)記載の塩基配列に基づいた制限酵素サイトを確認することによって行

った。

【0034】(実施例2) HGP-30遺伝子の合成とp37遺伝子およびHGP-30遺伝子を有する第二の組み換えプラスミドの作製

(1) p37遺伝子の後半部分を含んだプラスミドpGH37の作製(図2参照)

参考例1で得たpHP37を制限酵素HpaIと制限酵素BglIIで切断し、p37遺伝子の後半部分約1260bpのDNA断片を回収した。この断片をクレノウ処理し、平滑末端とした後、制限酵素DraIで完全消化したpBR322(Cold Spring Harbor Symposium、43、77、(1979))へ挿入し、組み換えプラスミドpGH37を得た。pGH37は、p37遺伝子の終止コドンに重なった位置にただひとつのDraIサイトを有している。

【0035】(2) pGH37へのHGP-30遺伝子に相当する合成DNAの挿入によるプラスミドpGH37HGPの作製(図2参照)

HGP-30遺伝子をコードするDNA(配列番号1)をDNA合成機を用いて合成した。この合成DNAは、中央部にHindIIIサイトを持つ。上記(1)で得たpGH37遺伝子を制限酵素DraIで処理し、先に得たHGP-30をコードする合成DNAを挿入し、組み換えプラスミドpGH37HGPを得た。pGH37HGPを制限酵素SspIで処理してp37遺伝子とHGP-30遺伝子の間付近のDNA断片を得、この断片の塩基配列をメッシングらの方法(Nucl. Acid Res.、9、309-321(1981))によって確認したところ、pGH37HGPを制限酵素DraIで処理したために、p37遺伝子とその下流にある

HGP-30遺伝子の間には終止コドンは消失し、チロシンをコードするコドンになったことがわかった。

【0036】(3) p37遺伝子後方周辺配列、およびp37遺伝子前半配列の付加による組み換えプラスミドpKPHGP30の作製(図3、4参照)

上記(2)で得たpGH37HGPを制限酵素HindIIIと制限酵素SspIで切断し、HGP-30遺伝子の後半部分を含む361bpのDNA断片を回収した。また、上記(1)で得たpHP37を制限酵素PstIと制限酵素SspIで切断し、p37遺伝子の後方配列を含む約1250bpのDNA断片を回収した。次に、pUC18を制限酵素HindIIIと制限酵素PstIで処理し、回収した361bpのDNA断片と約1250bpのDNA断片を挿入し、組み換えプラスミドpHPRearを作製した。

【0037】同様に、上記(2)で得たpGH37HGPを制限酵素HindIIIと制限酵素SspIで切断し、HGP-30遺伝子の前半部分を含む91bpのDNA断片を回収した。また、上記(1)で得たpHP37を制限酵素KpnIと制限酵素SspIで切断し、p37遺伝子の前方配列を含む約1050bpのDNA断片を回収した。次に、pUC18を制限酵素HindIIIと制限酵素KpnIで処理し、回収した91bpのDNA断片と約1050bpのDNA断片を挿入し、組み換えプラスミドpHKFrontを作製した。

【0038】このようにして得た組み換えプラスミドpHPRearを制限酵素PstIと制限酵素HindIIIで切断し、HGP-30遺伝子の後半部分およびp37遺伝子の後方周辺部分の配列を含む約1510bpのDNA断片を回収した。また、組み換えプラスミドpHKFrontを制限酵素PstIと制限酵素HindIIIで切断し、HGP-30遺伝子の前半部分およびp37遺伝子の前方部分の配列を含む約1140bpのDNA断片を回収した。pUC18を制限酵素PstIと制限酵素KpnIで処理し、回収した二つの断片を挿入し、組み換えプラスミドpKPHGP30を得た。

【0039】(4) p37遺伝子前方配列の付加による第2の組み換えプラスミドpHPHGP30の作製(図5参照)

上記(3)で得た組み換えプラスミドpKPHGP30を制限酵素PstIと制限酵素KpnIで切断し、p37遺伝子後方周辺部分配列およびp37遺伝子を含む約2650bpのDNA断片を回収した。

【0040】また、上記(1)で得た組み換えプラスミドpHP37を制限酵素KpnIと制限酵素HindIIIで切断し、p37遺伝子の前方周辺部分配列を含む約1890bpのDNA断片を回収した。pUC18を制限酵素PstIと制限酵素HindIIIで処理し、回収した二つの断片を挿入し、組み換えプラスミドpPHGP30を作製した。

【0041】(実施例3) 第2の組み換えプラスミドpHPHGP30へのβ-ガラクトシダーゼ遺伝子の組み込みによる第3の組み換えプラスミドpHPHGP30Zの作製(図6参照)

(1) ワクチニアウイルス7.5Kプロモーター下流へのβ-ガラクトシダーゼ遺伝子の組み込んだpNZ76の作製

β-ガラクトシダーゼ遺伝子は、Gene. 28, 127-132 (1984) 記載のプラスミドpMA001を制限酵素BamHIで切断し回収した。7.5Kプロモーターの下流にポリリンカーをつないだプラスミドpAK8(特開昭64-74982号公報)をBamHIで処理し、先に回収したβ-ガラクトシダーゼ遺伝子を挿入し、組み換えプラスミドpNZ76を得た。

【0042】(2) 第2の組み換えプラスミドpHPHGP30へのβ-ガラクトシダーゼ遺伝子の組み込みによる第3の組み換えプラスミドpHPHGP30Zの作製

上記(1)で得たpNZ76を制限酵素HindIIIと制限酵素SmaIで切断し、7.5Kプロモーターとβ-ガラクトシダーゼ遺伝子のつながったDNA断片を回収し、さらにクレノウ処理し、約4KbpのDNA断片を得た。前述の参考例2(4)で得た組み換えプラスミドpHPHGP30を制限酵素SnaBIで処理し、先に得た約4KbpのDNA断片を挿入し、組み換えプラスミドpHPHGP30Zを作製した。

【0043】

(実施例4) 組み換えワクチニアウイルスの作製

25cm²のカルチャーボトルに培養されたRK-13細胞にワクチニアウイルスWR株を0.1p. f. u./細胞の割合で接種し、45分後、10μgの実例3で得た組み換えプラスミドpHPHGP30Zを2.2mlの滅菌水に溶解し、極高ら(蛋白・核酸・酵素、27, 340-(1985))の方法によってDNA-リン酸カルシウム共沈物をつくり、その0.5mlを感染RK-13細胞に滴下した。30分間、37℃、7%CO₂インキュベーターに静置し、5%牛胎児血清を含むイーグルMEM4.5mlを加えた。その3時間後、培養液を交換し、48時間、37℃、7%CO₂インキュベーター中で培養し、培養細胞毎に3度凍結融解し、組み換え体を含むウイルス液を得た。

【0044】組み換え体の選択のため、10cmベトリ皿に培養されたRK-13細胞に上記のウイルス液を接種し、30分後、0.8%アガロース、5%牛胎児血清を含むイーグルMEMを積層し、3日間培養後、感染細胞に0.8%アガロース、0.5mg/mlのブルオギアル(BRL社製)を含むイーグルMEMを積層し、14時間後に青色に染まったブラックからパスツールピペットでウイルスを抜き取り、これを2%ゼラチンを含むリン酸緩衝生理食塩水(以下、PBSという)に懸濁

し、一部はドットハイブリダイゼーションをするため、ナイロンまたはニトロセルロースメンブレンにスポットし、残りは-20℃で保存した。

【0045】スポットしたメンブレンは、0.5N水酸化ナトリウム水溶液で10分間、1Mトリス塩酸緩衝液で5分間の処理を後、1.5M塩化ナトリウム、0.5Mトリス塩酸緩衝液で5分間処理した。2倍SSC(1倍SSCは、0.15M塩化ナトリウム、0.015Mクエン酸ナトリウムを含む)で飽和させ、80℃、2時間焼き付けた。

【0046】その後、これを4倍SET(0.6M塩化ナトリウム、0.08Mトリス塩酸、4mMのEDTA、pH7.8)-10倍Denhardt-0.1%SDSからなる混合液で68℃、2時間処理した。4倍SET-10倍Denhardt-0.1%SDS-0.1%Na₂P₂O₇-50μg/ml変性サケ精子DNAとカイネーションによって³²Pで標識したHGP-30合成遺伝子を入れて、68℃、14時間ハイブリダイゼーションした。洗浄後、メンブレンとX線フィルムを重ね、オートラジオグラフィを行い、フィルムが黒化するスポットを選択した。

【0047】黒化したスポットに対応するウイルス液(ウイルス量m. o. i. = 3×10⁻⁶)を再度RK-13細胞に接種し、30分後、0.8%アガロース、5%牛胎児血清を含むイーグルMEMを積層し、3日間培養後、さらに0.8%アガロース、0.5mg/mlのブルオギアルを含むイーグルMEMを積層し、14時間後に青色に染まったブラックについて、上記と同様の操作を行い、出現するブラックがすべてドットハイブリダイゼーションで黒化するまで純化を繰り返した。こうして得られたウイルスは、目的の組み換えワクチニアウイルスであり、これをv37HGP30と命名した。

【0048】(比較例1) p37遺伝子を有さず小型エプトープHGP-30遺伝子を有する組み換えワクチニアウイルスの構築

(1) ウイルスの構造タンパク質p37遺伝子を有さず小型エプトープHGP-30遺伝子を有する組み換えプラスミドの作製(図3参照)

pAK8を制限酵素SalIと制限酵素EcoRIで処理し、DNA合成機で合成した塩基配列(配列番号2、5'上流側にSalIサイトを有し、3'下流側にEcoRIサイトを有する)を持つHGP-30合成遺伝子を挿入し、組み換えプラスミドpAK8HGP30を作製した。pAK8HGP30では、ワクチニアウイルスTK遺伝子が破壊され、ワクチニアウイルス7.5Kプロモーターの下流に、開始コドンと終止コドンを前後に有するHGP30合成遺伝子が結合された状態となっている。

【0049】(2) 組み換えワクチニアウイルスの構築
組み換えプラスミドとしてpHPHGP30Zの代わり

に、上記(1)で得た組み換えプラスミドpAK8HGP30を使用し、5-プロモ-2'-デオキシウリジンでウイルスを選択すること以外は、実施例4と同様の方法で組み換えワクチニアウイルスを構築し、得られた組み換えワクチニアウイルスをvTKHGP30と命名した。

【0050】(実施例5) 組み換えワクチニアウイルスの感染細胞での発現

組織培養用チャンバースライド上で5%牛胎児血清を含むイーグルMEMで増殖させたRK-13細胞に、m.o.i.=1の本発明の組み換えワクチニアウイルスv37HGP30、または比較例で構築したvTKHGP30をそれぞれ接種し、37℃で1時間放置後、それぞれウイルス液をのぞき、細胞をイーグルMEMで洗浄し、5%牛胎児血清を含むイーグルMEMを加えた。16時間後、イーグルMEMを除き、軽くPBSで洗い、風乾後室温で5分間アセトン処理し、細胞をそれぞれ固定した。

【0051】一次抗体として抗HGP-30モノクローナル抗体(バイラルテクノロジーズインク社製)、二次抗体としてイソチアン酸フルオレセイン結合抗マウスIgG抗体(TAGO社製)を使用する間接蛍光抗体法により、蛍光顕微鏡を用いて蛍光による特異的な発現を観察した。その結果、本発明の組み換えワクチニアウイルスv37HGP30は、多量の小型エビトープHGP-30を発現しているが、比較例の構造タンパク質遺伝子を有さない組み換えワクチニアウイルスvTKHGP30は、小型エビトープHGP-30を全く発現していないことがわかった。

【0052】(実施例6) ウェスタンブロッティングを用いた組み換えワクチニアウイルス感染細胞でのハイブリッドタンパク質の発現の確認
10cm²の培養プレートに5%牛胎児血清を含むイーグルMEMを用いて増殖させたRK-13細胞に、m.o.i.=1の実施例4で構築した組み換えワクチニアウイルスv37HGP30またはワクチニアウイルスWRommo株をそれぞれ接種し、37℃で1時間放置後、それぞれウイルス液をのぞき、細胞をイーグルMEMで洗浄し、5%牛胎児血清を含むイーグルMEMを加*

配列

ATAGCGTAC ATCAAAGGAT AGATGTAAAA GACACCAAGG AAGCTTTAGA GAAGATAGAG 60
GAAGAGCAA AACAAAAGTA AGAAAAAGGC TTA 94

【0055】

【配列表】配列番号:2

配列の長さ:104

配列の型:核酸

配列

TCGACATGT ATAGCGTACA TCAAAGGAIA GATGTAAAAG ACACCAAGGA AGCTTTAGAG 60
AAGATAGAG GAAGAGCAAA ACAAAGTAA GAAA
AAGGCT TAAG 104

*えた。16時間後、イーグルMEMを除き、軽くPBSで洗い、遠心管に移して3000rpmで5分間遠心分離し細胞を回収した。

【0053】次に、100μlのリシスバッファー(Nature, 227, 680(1970))を加えて再び細胞を懸濁した後、100℃で5分間処理した。次に15,000rpmで10分間の遠心分離で得られた上清をサンプルとし常法に従ってウェスタンブロッティングを行った。なお、使用した一次抗体は実施例5で用いたものと同じである。ウェスタンブロッティングの結果、v37HGP30感染細胞では、約40Kdのはっきりしたバンドが認められたのに対し、親株として用いたワクチニアウイルスは全くバンドが認められなかった。このことにより、v37HGP30感染細胞においてp37タンパク質にHGP-30タンパク質が付加されたハイブリッドタンパク質が発現していることがわかった。

【図面の簡単な説明】

【図1】組み換えプラスミドpHP37の作製方法を示した説明図である。

【図2】組み換えプラスミドpGH37HGPの作製方法を示した説明図である。

【図3】組み換えプラスミドpHPrearと組み換えプラスミドpHKFrontの作製方法を示した説明図である。

【図4】組み換えプラスミドpKPHGP30の作製方法を示した説明図である。

【図5】組み換えプラスミドpHPHGP30の作製方法を示した説明図である。

【図6】組み換えプラスミドpHPGHGP30Zの作製方法を示した説明図である。

【0054】

【配列表】配列番号:1

配列の長さ:94

配列の型:核酸

鎖の数:二本鎖

トポロジー:直鎖状

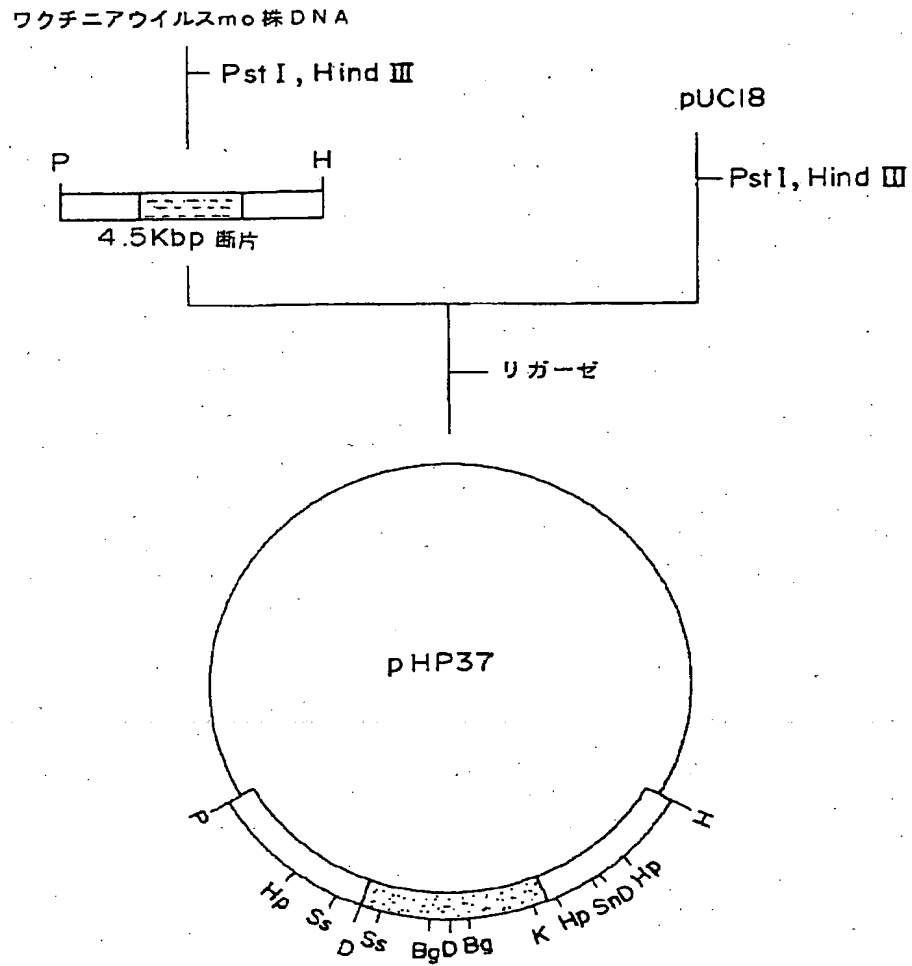
配列の種類:他の核酸 合成DNA

鎖の数:二本鎖

トポロジー:直鎖状

配列の種類:他の核酸 合成DNA

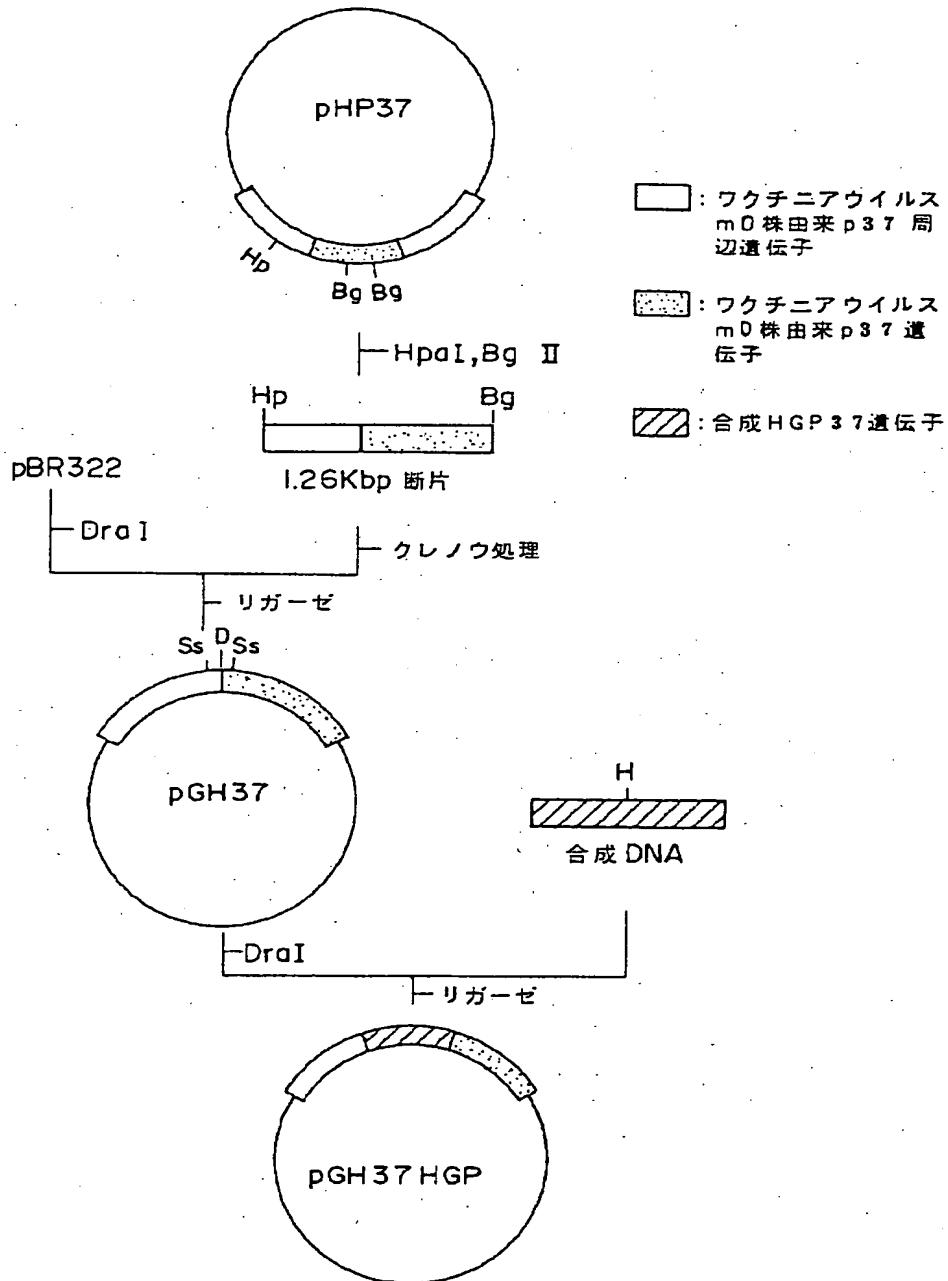
【図1】



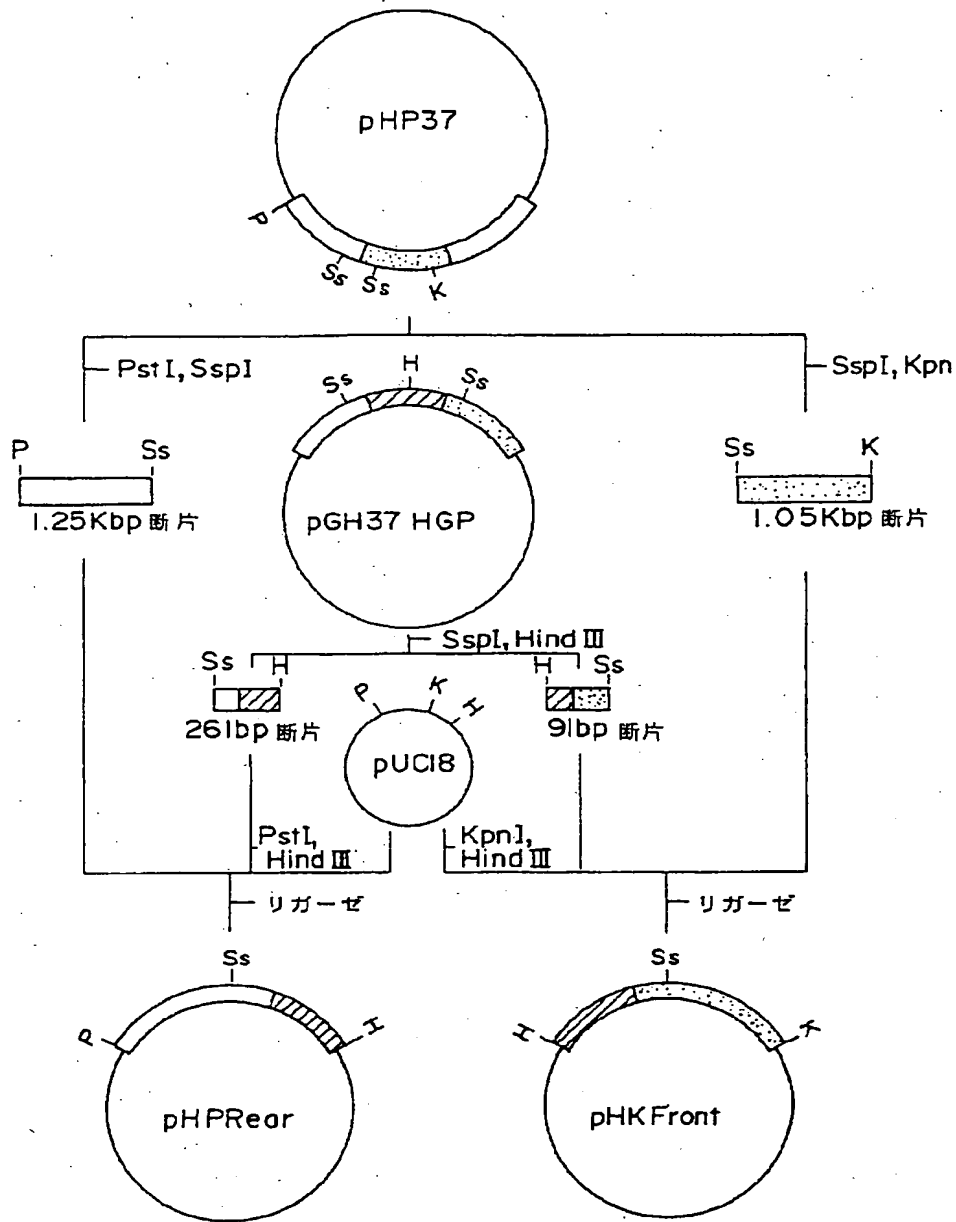
□ : ワクチニアウイルスm0株由来p37周辺遺伝子

■ : ワクチニアウイルスm0株由来p37遺伝子

【図2】



【図3】

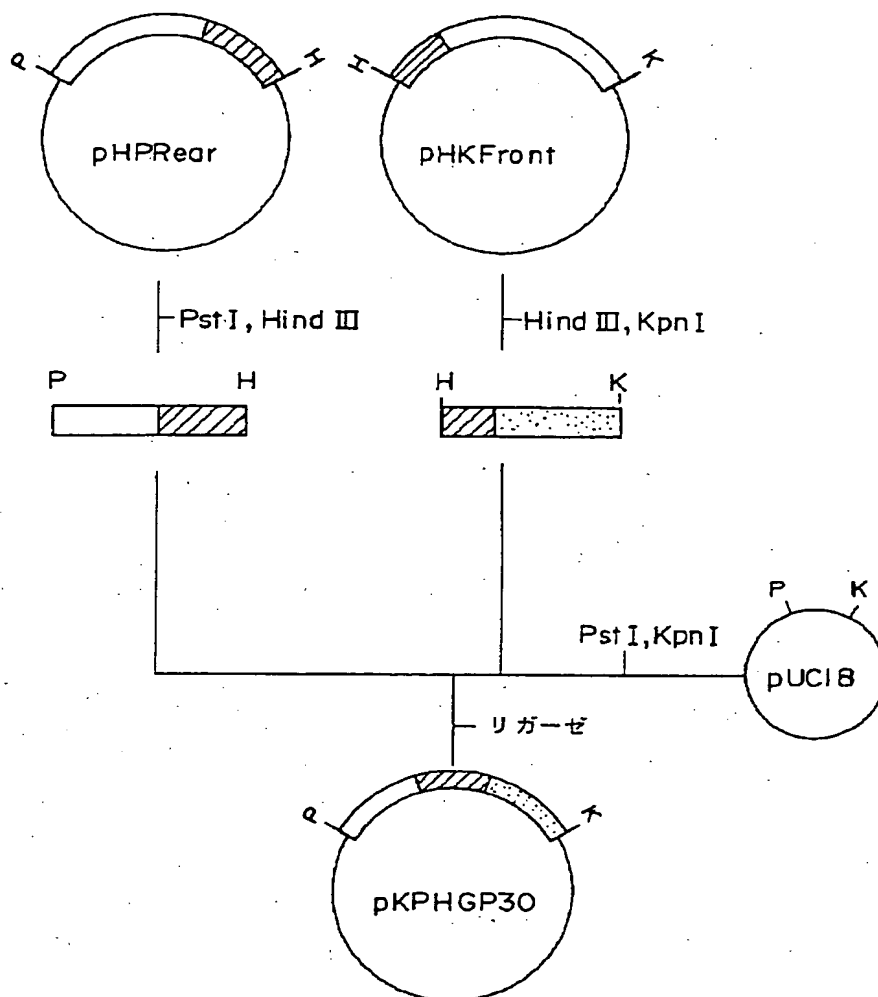


□: ワクチニアウイルスm0株由来p37周辺遺伝子

▨: ワクチニアウイルスm0株由来p37遺伝子

▧: 合成HGP37遺伝子

【図4】

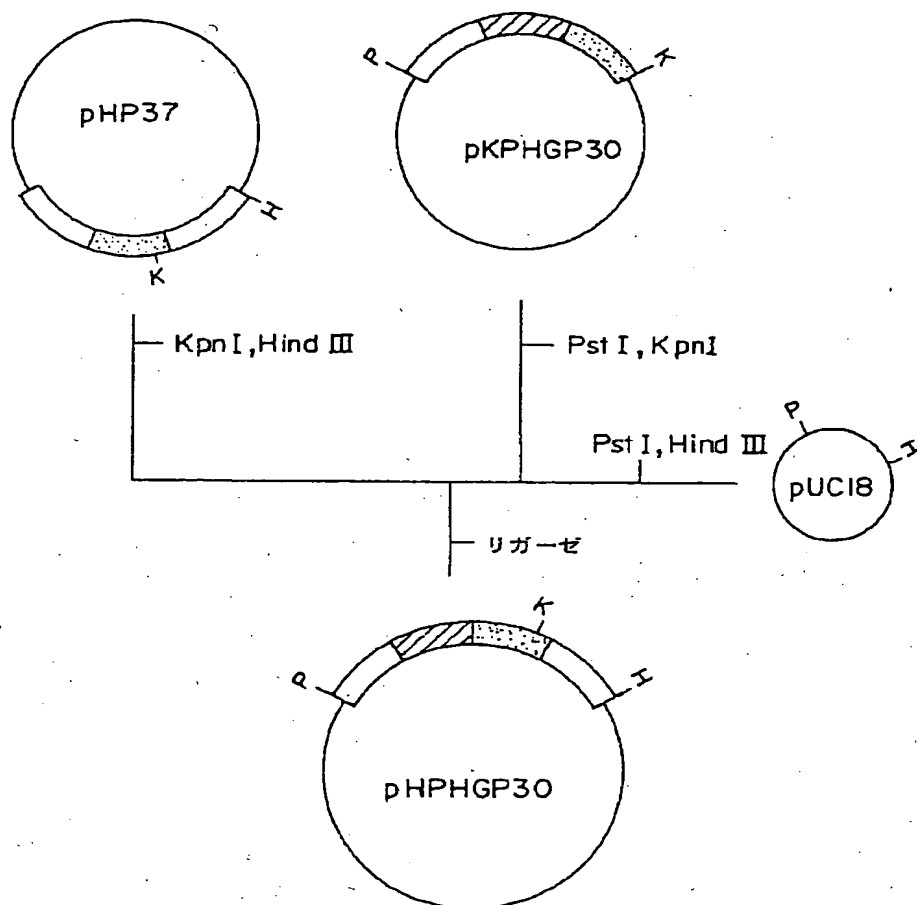


□ : ワクチニアウイルスm0株由来p37周辺遺伝子

□ : ワクチニアウイルスm0株由来p37遺伝子

▨ : 合成HGP37遺伝子

【図5】

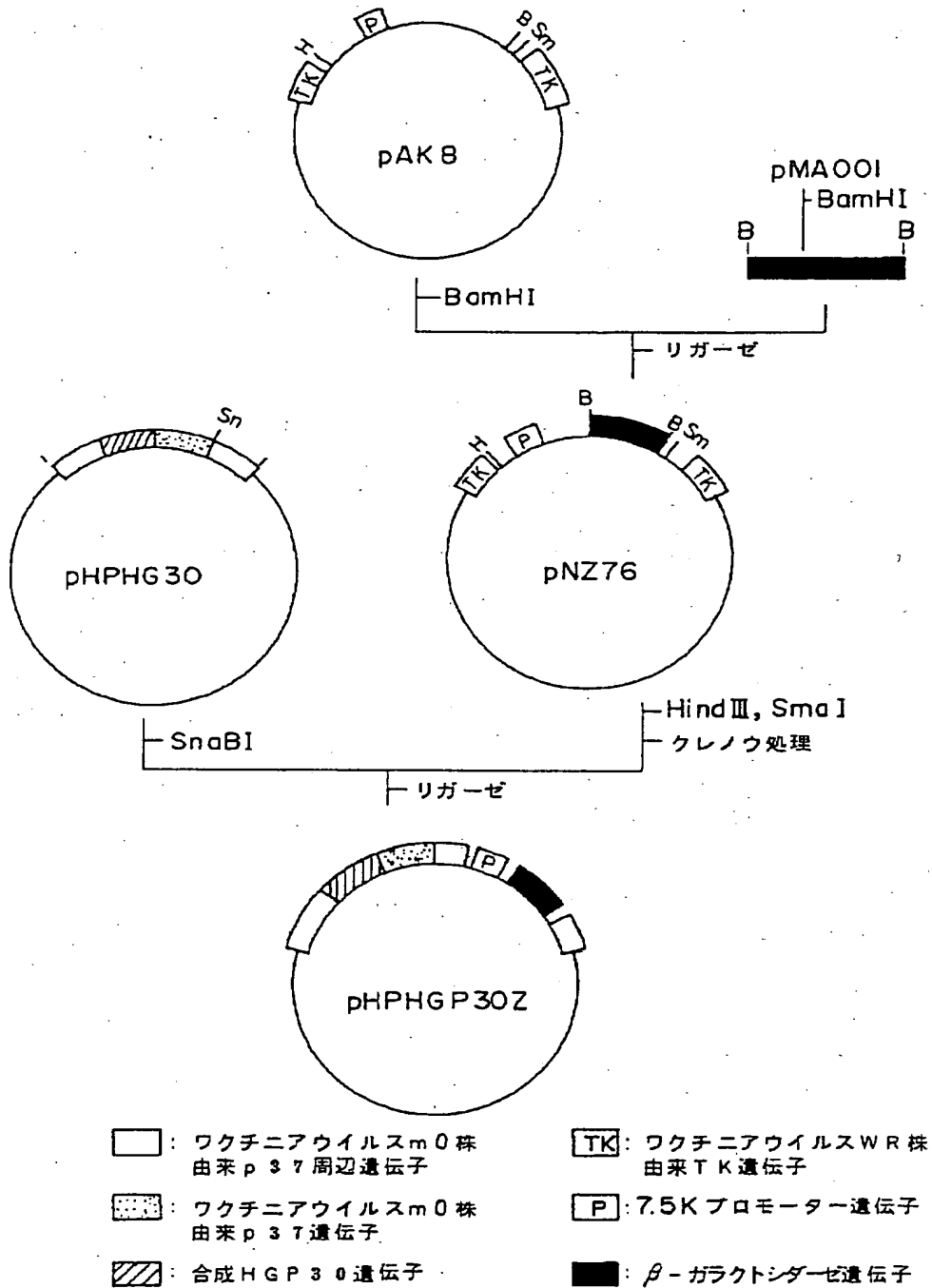


□ : ワクチニアウイルスm0株由来p37周辺遺伝子

▨ : ワクチニアウイルスm0株由来p37遺伝子

▧ : 合成HGP37遺伝子

【図6】



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(54) HYBRID ANTIGEN PROTEIN, RECOMBINED VIRUS FOR EXPRESSING THE SAME, AND ITS PRODUCTION

(57)Abstract:

PURPOSE: To provide the hybrid antigen protein having a virus-constituting protein and the small peptide epitope of a pathogenic virus and useful as a vaccine or a diagnostic medicine.

CONSTITUTION: The hybrid antigen protein having the virus-constituting protein and the small peptide epitope of the pathogenic virus is produced by preparing a recombined virus having the gene of the virus-constituting protein and the gene of the small peptide epitope of the pathogenic virus and subsequently allowing the recombined virus to express in the cultured cell.

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CLAIMS

[Claim(s)]

[Claim 1] Hybrid antigen protein which has the peptide epitope of the pathogenic virus whose structure protein and configuration amino acid of a virus are 300 or less.

[Claim 2] The recombination virus which included the gene which carries out the code of the peptide epitope of the pathogenic virus the gene which carries out the code of the structure protein of a virus, and whose configuration amino acid are 300 or less in propagation of a virus to the indispensable field.

[Claim 3] The manufacture technique of the protein according to claim 1 using the recombination virus according to claim 2.

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DETAILED DESCRIPTION

[Detailed Description of the Invention]

[0001]

[Field of the Invention] this invention relates to the manufacture technique of the hybrid antigen protein which has the peptide epitope of the pathogenic virus whose structure protein and configuration amino acid of a virus are 300 or less, the recombination virus which discovers it, and the hybrid antigen protein using it.

[0002]

[Description of the Prior Art] In antigen protein, such as a pathogenic virus various in recent years, bacteria, and a protozoa The peptide epitope of a fraction important for the phylaxis is found out. As a vaccine To use is tried (R.). Arnon] and M.Shapira — Modern Approaches (R.) to Vaccines, Molecular and Chemical Basis of Virus and Immunogenicity [M.Chenock] et.al.eds.p.109, Cold Spring Harbor (1984) . However, comparatively, generally, since an antigenicity was low, on these, it was made to combine with a Keyhole limpet hemocyanin etc., and the peptide vaccine of a short chain is used combining an adjuvant, and had the fault that side effects, such as inflammation by the adjuvant, happened.

[0003] It is reported that the antigenicity which whose safety was [the live vaccine by the recombination virus] also high, and was excellent on the other hand is shown (JP,64-74982,A etc.). Then, if the peptide epitope which is equivalent to a synthetic-peptide vaccine with a recombination virus can be made to discover efficiently, the antigenicity of the above-mentioned peptide vaccine will be considered that the fault of being low is improvable.

[0004] However, in order according to the experiment of this invention persons to rearrange a small peptide epitope and to be discovered using a virus, even if it used the technique (JP,64-74982,A official report etc.) of discovering large-sized antigen protein as usual as they were, a problem is in the stability within the cell of a small peptide etc., and sufficient amount of manifestations was not able to be obtained.

[0005]

[Problem(s) to be Solved by the Invention] Then, as a result of advancing a study zealously under such conventional technique that the peptide epitope of a short chain should be made to discover comparatively, this invention persons find out that the hybrid antigen protein incorporating the gene which carries out the code of the structure protein of a virus, and the gene which carries out the code of the peptide epitope of a pathogenic virus which has the structure protein and the small epitope of a virus efficiently when it rearranges and a virus is used is obtained, and came to complete this invention.

[0006]

[Means for Solving the Problem] According to this invention, 10-150, and the hybrid antigen protein (henceforth hybrid protein) that has more preferably 15-100, and the peptide epitope (henceforth a small epitope) of the pathogenic virus which are 20-50 still preferably are preferably offered [the structure protein (henceforth structure protein) and the configuration amino acid of a virus] for configuration amino acid 300 or less as the 1st invention in this way.

[0007] moreover, the gene (henceforth a structure protein gene) which carries out the code of the structure protein of the virus which discovers the hybrid protein as the 2nd invention, and configuration amino acid included the gene (henceforth a small epitope gene) which carries out the code of the peptide epitope of the pathogenic virus which is 300 or less in propagation of a virus to the un-indispensable field — it rearranges and a virus is offered Furthermore , the manufacture technique of hybrid protein using this recombination virus as the 3rd invention is offered.

[0008] A vaccinia virus, a baculovirus, a loon poxvirus, etc. are mentioned that the virus (henceforth a recipient virus) with which rearranges in this invention and production of a virus is presented should just use the virus used for general transgenics technique. What is necessary is it to be desirable to use the virus infected with the same kind as the animal with which the pathogenic virus used as the origin of a peptide epitope is infected, for example, just to use the vaccinia virus infected with the Homo sapiens, if the peptide epitope of the human immunodeficiency virus (henceforth HIV) origin is incorporated.

[0009] As an example of a vaccinia virus, WR stock (J. (Virol., 49 and 857, 1984)), The Lister stock, a temperature-sensitivity Lister stock (JP,62-44178,A), New York Board of Variolation vaccine stocks, such as Health stock and eight stocks of LC16m, are illustrated. as an example of a baculovirus Autographa potash ***** (Autographa californica), ***** ** (Trichoplusia ni), ***** **** (Rachiplusia ou), ***** ***** (Galleriamellonella), ***** **** (Bombyx mori), etc. are mentioned. as an example of a loon poxvirus ATCC VR-251, ATCC VR-250, ATCC VR-229, ATCC VR-249, ATCC VR-228, the Nishi-Gahara stock, NP stock (***** Nakano system stock), etc. are mentioned.

[0010] Although the structure protein gene used in this invention will not be limited especially if it functions as structure protein of a virus, the vaccinia virus main outer-membrane antigen p37 (J. Virol., 39,903 (1981)), a vaccinia virus hemagglutinin (Virology, 150, 451 (1986)), vaccinia virus Ag35 antigen (J. Virol., 181, 671 (1991)), etc. are mentioned, for example. Moreover, these protein genes could be embellished as long as what functions as structure protein was discovered.

[0011] When it considers from the point of an immunogenicity among such protein genes, the outer-membrane protein of a virus is desirable and what is outer-membrane protein of a virus and is discovered on the surface of an infected cell is more desirable. As an example of such a gene, vaccinia virus main outer-membrane protein p37 (J. Virol., 58,757 (1986)), a vaccinia virus hemagglutinin, etc. are mentioned. Moreover, it is more desirable to use the structure protein of the virus to incorporate and the virus belonging to the same group.

[0012] Although it will not be limited specially since it is uninfluential to propagation of a recipient virus if the size of the structure protein gene to incorporate is the virus of the same kind as the virus (henceforth a recipient virus) which the virus used as the origin of structure protein incorporates, when using a recipient virus and the structure protein gene of the virus origin of a different kind, it must be the size which is the grade which can increase a recipient virus, and a not much bigger gene cannot be incorporated. 10,000 or less bases of the sizes of the gene in such a case are usually 2,000 or less bases preferably.

[0013] The gene which carries out the code of the small epitope used in this invention is the epitope of a pathogenic virus and configuration amino acid is 10-150, and the gene that carries out the code of 15-100, and the thing that is 20-50 still preferably more preferably preferably 300 or less. for example, the gene (Proc.Natl.Acad.Sci.USA --) which carries out the code of HGP30 of HIV 84, 2951-2955 (1988), the gene (Proc.Natl.Acad.Sci.USA, 85, 1932 (1988)) that carries out the code of V3 of HIV are mentioned.

[0014] Moreover, these protein genes may be compounded, even if it could be embellished as long as what functions as a small epitope was discovered, and it is obtained from a further natural virus (the deficit of amino acid, an increase, and change are included) and it is a part of cDNA of a virogene.

[0015] It rearranges, a virus is produced and the hybrid protein of this invention is manufactured by [which included an above-mentioned configuration protein gene and an above-mentioned small epitope gene in the above-mentioned recipient virus] making this discover within a suitable host cell. As an example of such hybrid protein, the hybrid protein of vaccinia virus origin p37 protein and HIV origin HGP30 protein, the hybrid protein of vaccinia virus origin p37 protein and HIV origin V3 protein, etc. are mentioned.

[0016] Below, the general production technique of the recombination virus of this invention and hybrid protein is explained.

[0017] (Production of the 1st recombination vector) The 1st recombination vector contains the configuration protein gene used in order to produce the hybrid protein made into the purpose. When inserting a configuration protein gene and a small epitope gene in a recipient virus by homology recombination, a part of un-indispensable gene needs to be in propagation of a recipient virus to the ends of a structure protein gene. Therefore, when the virus to incorporate is a virus of the kind different from a recipient virus, it is necessary to make a part of un-indispensable gene field exist in propagation of the virus which serves as a recipient to the ends of a configuration protein gene.

[0018] although especially the vector used as the material of production of the 1st recombination vector is not limited -- pBR322, pBR325, pUC18, etc. -- the time -- a plasmid, a lambda phage, M13 phage, etc. -- the time -- a phage, pHC79 ((Gene, 11 and 291, 1980)), etc. -- the time -- a cosmid -- illustrating -- having .

[0019] Production of the 1st recombination vector can be performed according to a conventional method (JP,62-44178,A, JP,1-285198,A, JP,1-168279,A, etc.). For example, what is necessary is to start the part and its circumference section of the vaccinia virus configuration protein gene which uses DNA of the vaccinia virus origin prepared according to the technique of J.Virol.Methods and 2,175 to 179 (1981) publication by the suitable restriction enzyme, and just to include in a suitable vector, when using a vaccinia virus as the thing of the vaccinia virus origin, and a recipient virus as configuration protein.

[0020] (Production of the 2nd recombination vector) The small epitope gene made into the purpose is inserted or added to the fraction of ***** of the structure protein gene in the 1st recombination vector which carried out in this way and was produced, and the 2nd recombination vector is produced. It must be designed so that both may be tied by the reading frame of the right amino acid translation, a structure protein gene and a small epitope gene can also contain a promoting-agent fraction in an insertion or addition and both genes [neither of] may be destroyed.

[0021] When [of the start codon of a structure protein gene] adding a small epitope gene to the upstream immediately, in the style of [of a small epitope gene] the best, it is required, and a start codon needs to make a stop codon in the style of [of elimination and small epitope gene of the stop codon of a structure protein gene] the lowest, when [of the stop codon of a structure protein gene] adding a small epitope gene to a lower stream of a river immediately.

[0022] (Production of the 3rd recombination vector) The marker gene for making selection of a recombination virus easy is inserted by the conventional method to the 2nd recombination vector, and the 3rd recombination vector is produced.

[0023] Although especially a marker gene is not limited, a beta-galactosidase gene (Molecular And Cellular Biology, 5, 3403 (1985)), Ecogpt gene (J. Virol., 62, 1849-1854 (1988)), etc. are illustrated, for example. A marker gene is used in the status that it tied to the promoting agent's lower stream of a river.

[0024] As promoting agent who ties to a marker gene As what it will not be limited especially if it functions within the virus to incorporate, for example, is discovered within a vaccinia virus The promoting agent of the vaccinia virus gene which carries out the code of the 7.5K polypeptide The promoting agent of the vaccinia virus gene which carries out the code of (it is hereafter called 7.5K promoting agent) and the 19K polypeptide As what the promoting agent of the vaccinia virus gene which carries out the code of (it is hereafter called 19K promoting agent) and the 11K polypeptide etc. is illustrated, and is discovered within a baculovirus The promoting agent of the baculovirus gene which carries out the code of the ***** drine compounds of a baculovirus, As what the promoting agent of the baculovirus gene which carries out the code of the 10K polypeptide of a baculovirus etc. is illustrated, and is discovered within a loon poxvirus The promoting agent of the loon poxvirus gene which carries out the code of the thymidine kinase of a loon poxvirus, 7.5K promoting agent, 19K promoting agent, etc. are illustrated.

[0025] the marker gene tied to the promoting agent's lower stream of a river is boiled near [where the 2nd small epitope gene was inserted or added] the vaccinia virus configuration protein gene, it is inserted, and the 3rd recombination vector is produced The system of the easy Escherichia coli of a gene manipulation should just be used for construction this ***** of the these [1st], the 2nd, and 3rd recombination vectors.

[0026] (A construction of a recombination virus, and proteinic manufacture) Introduce into the cell which infected beforehand with the virus the 3rd recombination vector obtained by the above-mentioned technique, homology recombination is made to cause between vector DNA and the gene of a viral genome, and a recombination virus is built. If in charge of a construction of a recombination virus, that what is necessary is just to carry out according to a conventional method, the 3rd recombination vector is made to introduce in a virus by the calcium phosphate coprecipitation method, the liposome method, the microinjection, the electroporation method, etc., and the virus ensemble containing the recombination virus obtained is cultivated on a suitable culture medium.

[0027] A plaque is made to form by the technique suitable for the marker gene inserted by the 3rd recombination

vector, and the candidate stock of the target recombination vaccinia virus is obtained. What is necessary is to carry out multistep [of the MEM containing the halogenation indolyl-beta-D-galactosidase (henceforth a ***** gal), and the agar], and just to consider as the candidate stock of the recombination vaccinia virus aiming at the plaque dyed blue in a night as a marker gene, after making a plaque form, when a beta-galactosidase gene is used.

[0028] The technique of choosing the recombinant virus made into the purpose from these candidate stocks should just carry out the ***** assay using the antiserum or monoclonal antibody to the small epitope which purifies a plaque using the hybridization method which uses as a probe the small epitope gene made into the purpose, or is made into the purpose. Thus, hybrid protein will be produced, if the obtained recombination virus is infected with a host cell and cultivated.

[0029] The cell used here will not be limited especially if the virus which serves as a recipient is infected. TK-143 when the virus which serves as a recipient is a vaccinia virus (Homo-sapiens osteosarcoma origin), floor line (Homo-sapiens amnion origin), Hela (Homo-sapiens uterine cervix cancer origin), KB (Homo-sapiens nose throat cancer origin), valve flow coefficient-1 (ape kidney origin), BSC-1 (ape kidney origin), RK13 (lagomorph kidney origin), L929 (mouse connective-tissue origin), CE (****), CEF (hen fetus fibroblast), etc. are illustrated. When using a baculovirus, Sf9 cell of the Spodoptera full ***** (Spodoptera frugiperda) origin etc. is illustrated. When using a loon poxvirus, CE (****), CEF (hen fetus fibroblast), etc. are illustrated.

[0030] Thus, what is necessary is to collect and crush a cell and just to collect it, since the produced hybrid protein usually exists in large quantities in a host cell while the host cell is valid. Hybrid protein can be refined by adopting, combining suitably the fractionation method by a well-known conventional method, for example, a salting-out, a gel filtration, the ion exchange and the separation method by the affinity column chromatography, the high performance chromatography, and electrophoresis etc.

[0031]

[Effect of the Invention] In this way, according to this invention, a small epitope can be discovered as hybrid antigen protein with structure protein, and this protein can be used as an antigen of a component vaccine, and, as for a recombination virus, the use as live vaccine is expected.

[0032]

[Example] An example is raised to below and this invention is explained to it still concretely.

(Example 1) First, manufacture of the production (refer to drawing 1) vaccinia virus genomic DNA of vaccinia virus main outer-membrane antigen p37 gene and the 1st recombination plasmid pHP37 by the cloning of the circumference cultivated the vaccinia virus LC16mO stock (it is called a vaccinia virus mO stock clinical, a virus, 3(3)13-19 (1975), and the following) by the conventional method, and was performed by the technique of J.Virol.Methods and 2,175 to 179 (1981) publication.

[0033] This genomic DNA was cut by restriction enzyme PstI and restriction enzyme HindIII, and the DNA fragments of 4452bps containing p37 gene were collected. pUC18 was cut by restriction enzyme PstI and restriction enzyme HindIII, the DNA fragment obtained previously was inserted and the first recombination plasmid pHP37 was produced. authentication of pHP37 checks the restriction enzyme site based on the base sequence of Virol., 179, and 247-266 (1990) publication — it carried out

[0034] (Example 2) Production of the plasmid pGH37 containing the second half fraction of production (1) p37 gene of the second recombination plasmid which has synthesis of HGP-30 gene, p37 gene, and HGP-30 gene (refer to the drawing 2)

pHP37 obtained in the example 1 of reference was cut by restriction enzyme HpaI and restriction enzyme BglII, and the DNA fragments of second half partial abbreviation 1260bp of p37 gene were collected. After having carried out Klenow processing of this fragment and considering as a flush end, it inserted in pBR322 ((Cold Spring Harbor Symposium, 77 [43 and 77], 1979)) which carried out full digestion by restriction enzyme DraI, and the recombination plasmid pGH37 was obtained. pGH37 merely has one DraI site in the position which lapped with the stop codon of p37 gene.

[0035] (2) Production of plasmid pGH37HGP by insertion of the synthetic DNA equivalent to HGP-30 gene to pGH37 (refer to the drawing 2)

DNA (array number 1) which carries out the code of the HGP-30 gene was compounded using the DNA-synthesis machine. This synthetic DNA has HindIII site in a center section. pGH37 gene obtained above (1) was processed by restriction enzyme DraI, the synthetic DNA which carries out the code of the HGP-30 obtained previously was inserted, and recombination plasmid pGH37HGP was obtained. Process pGH37HGP by restriction enzyme SspI, and the DNA fragment near between p37 gene and HGP-30 genes is obtained. Since pGH37HGP was processed by restriction enzyme DraI when the base sequence of this fragment was checked by *****'s et al. technique (Nucl.Acids.Res., 9,309-321 (1981)) The stop codon disappeared between HGP-30 genes in p37 gene and its lower stream of a river, and it turns out that it became the codon which carries out the code of the tyrosine.

[0036] (3) p37 gene back circumference array and production of the recombination plasmid pKPHGP30 according to addition of an array the first half of p37 gene (drawing 3 , four references)

pGH37HGP obtained above (2) was cut by restriction enzyme HindIII and restriction enzyme SspI, and the DNA fragments of 361bps containing the second half fraction of HGP-30 gene were collected. Moreover, pHP37 obtained above (1) was cut by restriction enzyme PstI and restriction enzyme SspI, and the DNA fragments of about 1250 bps containing the back array of p37 gene were collected. Next, pUC18 was processed by restriction enzyme HindIII and restriction enzyme PstI, the DNA fragment of collected 361bps and the DNA fragment of about 1250 bps were inserted, and recombination plasmid pHPRear was produced.

[0037] pGH37HGP obtained above (2) was similarly cut by restriction enzyme HindIII and restriction enzyme SspI, and the DNA fragments of 91bps containing a part for the first portion of HGP-30 gene were collected. Moreover, pHP37 obtained above (1) was cut by restriction enzyme KpnI and restriction enzyme SspI, and the DNA fragments of about 1050 bps containing the front array of p37 gene were collected. Next, pUC18 was processed by restriction enzyme HindIII and restriction enzyme KpnI, the DNA fragment of collected 91bps and the DNA fragment of about 1050 bps were inserted, and recombination plasmid pHKFront was produced.

[0038] Thus, the obtained DNA fragments of about 1510 bps which rearranged, cut plasmid pHPRear by restriction enzyme PstI and restriction enzyme HindIII, and include the array of the second half fraction of HGP-30 gene and the back circumference fraction of p37 gene were collected. Moreover, recombination plasmid pHKFront was cut by restriction enzyme PstI and restriction enzyme HindIII, and the DNA fragments of about 1140 bps containing the array

of a part for the first portion of HGP-30 gene and the front fraction of p37 gene were collected. pUC18 was processed by restriction enzyme PstI and restriction enzyme KpnI, two collected fragments were inserted and the recombination plasmid pKPHGP30 was obtained.

[0039] (4) Production of the 2nd recombination plasmid pPHGP30 by addition of p37 gene front array (refer to the drawing 5)

The DNA fragments of about 2650 bps which rearrange, cut a plasmid pKPHGP30 by restriction enzyme PstI and restriction enzyme KpnI, and contain p37 gene back circumference partial array and p37 gene obtained above (3) were collected.

[0040] Moreover, the DNA fragments of about 1890 bps which rearrange, cut a plasmid pHP37 by restriction enzyme KpnI and restriction enzyme HindIII, and include the front circumference partial array of p37 gene obtained above (1) were collected. pUC18 was processed by restriction enzyme PstI and restriction enzyme HindIII, two collected fragments were inserted and the recombination plasmid pPHGP30 was produced.

[0041] (Example 3) Production of 3rd recombination plasmid pPHGP30Z by inclusion of the beta-galactosidase gene to the 2nd recombination plasmid pPHGP30 (refer to the drawing 6)

(1) The production beta-galactosidase gene of pNZ76 which the beta-galactosidase gene to a vaccinia virus 7.5K promoting-agent lower stream of a river incorporated cut and collected the plasmids pMA001 of Gene and 28,127 to 132 (1984) publication by restriction enzyme BamHI. The plasmid pAK8 (JP,64-74982,A) which connected the polylinker with 5K promoting agent's lower stream of a river was processed by BamHI, the beta-galactosidase gene collected previously was inserted and the recombination plasmid pNZ76 was obtained.

[0042] (2) pNZ76 obtained by the production above (1) of 3rd recombination plasmid pPHGP30Z by inclusion of the beta-galactosidase gene to the 2nd recombination plasmid pPHGP30 was cut by restriction enzyme HindIII and restriction enzyme SmaI, the DNA fragments with which 7.5K promoting agent and the beta-galactosidase gene were connected were collected, Klenow processing was carried out further, and the DNA fragment of about 4 Kbps was obtained. The DNA fragment of about 4 Kbps which processed the plasmid pPHGP30 by restriction enzyme SmaI by rearranging, and were obtained previously obtained in the above-mentioned example 2 of reference (4) was inserted, and recombination plasmid pPHGP30Z was produced.

[0043]

(Example 4) A vaccinia virus WR stock is inoculated into RK-13 cell cultivated by the culture bottle of 2.25cm of production of a recombination vaccinia virus at a rate of 0.1 p.f.u. / cell. Recombination plasmid pPHGP30Z obtained in the example 3 of 10microg is melted in a 2.2ml sterilized water 45 minutes after. By Hidaka's et al. (protein, a nucleic acid and an enzyme, 27,340- (1985)) technique, DNA-calcium phosphate coprecipitate was built and the 0.5ml was dropped at infection RK-13 cell. It put on 37 degrees C and 7% CO2 incubator gently for 30 minutes, and 4.5ml of the MEM which contains fetal calf serum 5% was added. Culture medium was exchanged 3 hours [the] after, the 3 times freeze thawing was cultivated and carried out for every cultured cell in 37 degrees C and 7% CO2 incubator, and the virus liquid containing the recombination field was obtained for 48 hours.

[0044] The above-mentioned virus liquid is inoculated into RK-13 cell cultivated by 10cm Petri dish for selection of the recombination field. The laminating of 0.8% agarose and the MEM which contains fetal calf serum 5% is carried out 30 minutes after. The laminating of the MEM which contains agarose and a 0.5mg [/ml] ***** gal (product made from BRL) in an infected cell 0.8% will be carried out after incubation for three days. In order that a virus may be sampled by the Pasteur pipette from the plaque which dyed blue 14 hours after, this may be suspended in the phosphate buffered saline (henceforth PBS) which contains gelatin 2% and a part may carry out a dot hybridization. The spot was carried out to nylon or the nitrocellulose membrane, and the remainder was saved at -20 degrees C.

[0045] the membrane which carried out the spot processed processing for 5 minutes for 5 minutes the back with 1M tris hydrochloric-acid buffer solution for 10 minutes in 0.5N sodium-hydroxide aqueous solution with 1.5M sodium chloride and 0.5M tris hydrochloric-acid buffer solution. You made it saturated with 2 double SSC (for SSC to contain 0.15M sodium chloride and 0.015M sodium citrate 1 time), and 80 degrees C was printed for 2 hours.

[0046] Then, this was processed 68 degrees C for 2 hours with the mixed liquor which consists of 4 times SET(0.6M sodium chloride, 0.08M tris hydrochloric-acid, EDTA [of 4mM], pH 7.8)-10 times Denhardt-0.1% SDS. By 4 times SET-10 times Denhardtseven to 50 microg [/ml] O [-0.1%SDS-0.1%Na4P2] denaturation salmon spermium DNA, and ***** by 32P, HGP-30 synthetic gene which carried out the indicator was put in, and the hybridization of the 68 degrees C was carried out for 14 hours. The membrane and the X-ray film were piled up after washing, autoradiography was performed, and the spot in which a film carries out a melanism was chosen.

[0047] The virus liquid (amount m.o.i.=of viruses3x10-6) corresponding to the spot which carried out the melanism is again inoculated into RK-13 cell. The laminating of 0.8% agarose and the MEM which contains fetal calf serum 5% is carried out 30 minutes after. The laminating of the MEM which contains agarose and a 0.5mg [/ml] ***** gal 0.8 more% will be carried out after incubation for three days. Purification was repeated until it performed the same operation as the above and all the appearing plaques carried out the melanism by the dot hybridization about the plaque which dyed blue 14 hours after. In this way, the obtained virus is the target recombination vaccinia virus, and named this v37HGP30.

[0048] (Example 1 of a comparison) Production of the recombination plasmid which does not have structure protein p37 gene of the construction (1) virus of a recombination vaccinia virus which does not have p37 gene but has small epitope HGP-30 gene, but has small epitope HGP-30 gene (refer to the drawing 3)

pAK8 was processed by restriction enzyme SalI and restriction enzyme EcoRI, HGP-30 synthetic gene with the base sequence (it has SalI site in array number 2 and 5' upstream side, and has EcoRI site in 3' lower-stream-of-a-river side) compounded with the DNA-synthesis machine was inserted, and recombination plasmid pAK8HGP30 was produced. In pAK8HGP30, a vaccinia virus TK gene is destroyed and it is in the status that HGP30 synthetic gene which has a start codon and a stop codon forward and backward on vaccinia virus 7.5K promoting agent's lower stream of a river was combined.

[0049] (2) as a construction recombination plasmid of a recombination vaccinia virus, instead of pPHGP30Z, it rearranges and plasmid pAK8HGP30 is used, except the thing which obtained above (1) and for which a virus is chosen with 5-*****-2'-deoxyuridine, it rearranges by the same technique as an example 4, a vaccinia virus is built, and it was obtained -- it rearranged and the vaccinia virus was named vTKHGP30

[0050] (Example 5) vTKHGP30 built in recombination vaccinia virus v37HGP30 or the example of a comparison of this invention of m.o.i.=1 was inoculated, respectively, virus liquid was removed into RK-13 cell proliferated by the MEM

which is in chamber sliding for manifestation tissue culture by the infected cell of a recombination vaccinia virus, and contains fetal calf serum 5% after 1 hour neglect at 37 degrees C, respectively, the cell was washed into it by MEM, and the MEM which contains fetal calf serum 5% was 16 hours after, except for MEM, it washed by PBS lightly, acetone processing was carried out for 5 minutes at the room temperature after air-drying, and the cell was fixed, respectively.

[0051] The specific issue by fluorescence was observed using the fluorescence microscope by the indirect fluorescent antibody technique which uses anti-HGP-30 monoclonal antibody (made in ***** Technologies, Inc.) as a primary antibody, and uses an iso ***** acid fluorescein joint anti-mouse IgG antibody (product made from TAGO) as a second antibody. Consequently, although recombination vaccinia virus v37HGP30 of this invention had discovered a lot of small epitope HGP-30, it rearranged, and the vaccinia virus vTKHGP30 completely discovered small epitope HGP-30, and it turns out [****] that it does not have the structure protein gene of the example of a comparison.

[0052] (Example 6) Into RK-13 cell proliferated using the MEM using the Western blotting which rearranges and contains fetal calf serum in the incubation plate of 2.5% 10cm of authentication of a manifestation of the hybrid protein in a vaccinia virus infected cell. Recombination vaccinia virus v37HGP30 or the vaccinia virus WRormo stock built in the example 4 of o.i.=1 is inoculated, respectively. Virus liquid was removed after 1 hour neglect at 37 degrees C, respectively, the cell was washed by MEM, and the MEM which contains fetal calf serum 5% was added. 16 hours after, except for MEM, it washed by PBS lightly, it moved to the centrifuging tube, the at-long-intervals core separation was carried out by 3000rpm for 5 minutes, and cells were collected.

[0053] Next, after having added the ***** buffer (Nature, 227, 680 (1970)) of 100microl and suspending a cell again, it processed for 5 minutes at 100 degrees C. Next, the supernatant liquid obtained by the centrifugal separation for 10 minutes by 15,000rpm was made into the sample, and the Western blotting was performed according to the conventional method. In addition, the used primary antibody is the same as that of what was used in the example 5. As for the vaccinia virus used as a parent strain, by v37HGP30 infected cell, a band was not accepted at all to the band with which about 40 Kds clarified having been accepted as a result of the Western blotting. It turns out that the hybrid protein with which HGP-30 protein was added to p37 protein in v37HGP30 infected cell is discovered with this.

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TECHNICAL FIELD

[Field of the Invention] this invention relates to the manufacture technique of the hybrid antigen protein which has the peptide epitope of the pathogenic virus whose structure protein and configuration amino acid of a virus are 300 or less, the recombination virus which discovers it, and the hybrid antigen protein using it.

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PRIOR ART

[Description of the Prior Art] In antigen protein, such as a pathogenic virus various in recent years, bacteria, and a protozoa The peptide epitope of a fraction important for the phylaxis is found out. As a vaccine To use is tried (R.). Arnon] and M.Shapira — Modern Approaches (R.) to Vaccines, Molecular and Chemical Basis of Virus and Immunogenicity [M.Chenock] et.al.eds.p.109, Cold Spring Harbor (1984) . However, comparatively, generally, since an antigenicity was low, on these, it was made to combine with a Keyhole limpet hemocyanin etc., and the peptide vaccine of a short chain is used combining an adjuvant, and had the fault that side effects, such as inflammation by the adjuvant, happened.

[0003] It is reported that the antigenicity which whose safety was [the live vaccine by the recombination virus] also high, and was excellent on the other hand is shown (JP,64-74982,A etc.). Then, if the peptide epitope which is equivalent to a synthetic-peptide vaccine with a recombination virus can be made to discover efficiently, the antigenicity of the above-mentioned peptide vaccine will be considered that the fault of being low is improvable.

[0004] However, in order according to the experiment of this invention persons to rearrange a small peptide epitope and to be discovered using a virus, even if it used the technique (JP,64-74982,A official report etc.) of discovering large-sized antigen protein as usual as they were, a problem is in the stability within the cell of a small peptide etc., and sufficient amount of manifestations was not able to be obtained.

[Translation done.]

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EFFECT OF THE INVENTION

[Effect of the Invention] In this way, according to this invention, a small epitope can be discovered as hybrid antigen protein with structure protein, and this protein can be used as an antigen of a component vaccine, and, as for a recombination virus, the use as live vaccine is expected.

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TECHNICAL PROBLEM

[Problem(s) to be Solved by the Invention] Then, as a result of advancing a study zealously under such conventional technique that the peptide epitope of a short chain should be made to discover comparatively, this invention persons find out that the hybrid antigen protein incorporating the gene which carries out the code of the structure protein of a virus, and the gene which carries out the code of the peptide epitope of a pathogenic virus which has the structure protein and the small epitope of a virus efficiently when it rearranges and a virus is used is obtained, and came to complete this invention.

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MEANS

[Means for Solving the Problem] According to this invention, 10-150, and the hybrid antigen protein (henceforth hybrid protein) that has more preferably 15-100, and the peptide epitope (henceforth a small epitope) of the pathogenic virus which are 20-50 still preferably are preferably offered [the structure protein (henceforth structure protein) and the configuration amino acid of a virus] for configuration amino acid 300 or less as the 1st invention in this way.

[0007] moreover, the gene (henceforth a structure protein gene) which carries out the code of the structure protein of the virus which discovers the hybrid protein as the 2nd invention, and configuration amino acid included the gene (henceforth a small epitope gene) which carries out the code of the peptide epitope of the pathogenic virus which is 300 or less in propagation of a virus to the un-indispensable field — it rearranges and a virus is offered Furthermore , the manufacture technique of hybrid protein using this recombination virus as the 3rd invention is offered.

[0008] A vaccinia virus, a baculovirus, a loon poxvirus, etc. are mentioned that the virus (henceforth a recipient virus) with which rearranges in this invention and production of a virus is presented should just use the virus used for general transgenics technique. What is necessary is it to be desirable to use the virus infected with the same kind as the animal with which the pathogenic virus used as the origin of a peptide epitope is infected, for example, just to use the vaccinia virus infected with the Homo sapiens, if the peptide epitope of the human immunodeficiency virus (henceforth HIV) origin is incorporated.

[0009] As an example of a vaccinia virus, WR stock (J. Virol., 49 and 857, 1984)), The Lister stock, a temperature-sensitivity Lister stock (JP,62-44178,A), New York Board of Variolation vaccine stocks, such as Health stock and eight stocks of LC16m, are illustrated. as an example of a baculovirus Autographa potash ***** (Autographa californica), ***** ** (Trichoplusia ni), ***** **** (Rachiplusia ou), ***** (Galleriamellonella), ***** **** (Bombyx mori), etc. are mentioned. as an example of a loon poxvirus ATCC VR-251, ATCC VR-250, ATCC VR-229, ATCC VR-249, ATCC VR-228, the Nishi-Gahara stock, NP stock (***** Nakano system stock), etc. are mentioned.

[0010] Although the structure protein gene used in this invention will not be limited especially if it functions as structure protein of a virus, the vaccinia virus main outer-membrane antigen p37 (J. Virol., 39,903 (1981)), a vaccinia virus hemagglutinin (Virology, 150, 451 (1986)), vaccinia virus Ag35 antigen (J. Virol., 181, 671 (1991)), etc. are mentioned, for example. Moreover, these protein genes could be embellished as long as what functions as structure protein was discovered.

[0011] When it considers from the point of an immunogenicity among such protein genes, the outer-membrane protein of a virus is desirable and what is outer-membrane protein of a virus and is discovered on the surface of an infected cell is more desirable. As an example of such a gene, vaccinia virus main outer-membrane protein p37 (J. Virol., 58,757 (1986)), a vaccinia virus hemagglutinin, etc. are mentioned. Moreover, it is more desirable to use the structure protein of the virus to incorporate and the virus belonging to the same group.

[0012] Although it will not be limited especially since it is uninfluent to propagation of a recipient virus if the size of the structure protein gene to incorporate is the virus of the same kind as the virus (henceforth a recipient virus) which the virus used as the origin of structure protein incorporates, when using a recipient virus and the structure protein in gene of the virus origin of a different kind, it must be the size which is the grade which can increase a recipient virus, and a not much big gene cannot be incorporated. 10,000 or less bases of the sizes of the gene in such a case are usually 2,000 or less bases preferably.

[0013] The gene which carries out the code of the small epitope used in this invention It is the epitope of a pathogenic virus and configuration amino acid is 10-150, and the gene that carries out the code of 15-100, and the thing that are 20-50 still preferably more preferably preferably 300 or less. for example, the gene (Proc.Natl.Acad.Sci.USA —) which carries out the code of HGP30 of HIV 84, 2951-2955 (1988), the gene (Proc.Natl.Acad.Sci.USA, 85, 1932 (1988)) that carries out the code of V3 of HIV are mentioned.

[0014] Moreover, these protein genes may be compounded, even if it could be embellished as long as what functions as a small epitope was discovered, and it is obtained from a further natural virus (the deficit of amino acid, an increase, and change are included) and it is a part of cDNA of a virogene.

[0015] It rearranges, a virus is produced and the hybrid protein of this invention is manufactured by [which included an above-mentioned configuration protein gene and an above-mentioned small epitope gene in the above-mentioned recipient virus] making this discover within a suitable host cell. As an example of such hybrid protein, the hybrid protein of vaccinia virus origin p37 protein and HIV origin HGP30 protein, the hybrid protein of vaccinia virus origin p37 protein and HIV origin V3 protein, etc. are mentioned.

[0016] Below, the general production technique of the recombination virus of this invention and hybrid protein is explained.

[0017] (Production of the 1st recombination vector) The 1st recombination vector contains the configuration protein gene used in order to produce the hybrid protein made into the purpose. When inserting a configuration protein gene and a small epitope gene in a recipient virus by homologous recombination, a part of un-indispensable gene needs to be in propagation of a recipient virus to the ends of a structure protein gene. Therefore, when the virus to incorporate is a virus of the kind different from a recipient virus, it is necessary to make a part of un-indispensable gene field exist in propagation of the virus which serves as a recipient to the ends of a configuration protein gene.

[0018] although especially the vector used as the material of production of the 1st recombination vector is not limited — pBR322, pBR325, pUC18, etc. — the time — a plasmid, a lambda phage, M13 phage, etc. — the time — a phage,

pHC79 ((Gene, 11 and 291, 1980)), etc. -- the tim -- a cosmid -- illustrating -- having .

[0019] Production of the 1st recombination vector can be performed according to a conventional method (JP,62-44178,A, JP,1-285198,A, JP,1-168279,A, etc.). For example, what is necessary is to start the part and its circumference section of the vaccinia virus configuration protein gene which uses DNA of the vaccinia virus origin prepared according to the technique of J.Virol.Methods and 2,175 to 179 (1981) publication by the suitable restriction enzyme, and just to include in a suitable vector, when using a vaccinia virus as the thing of the vaccinia virus origin, and a recipient virus as configuration protein.

[0020] (Production of the 2nd recombination vector) The small epitope gene made into the purpose is inserted or added to the fraction of ***** of the structure protein gene in the 1st recombination vector which carried out in this way and was produced, and the 2nd recombination vector is produced. It must be designed so that both may be tied by the reading frame of the right amino acid translation, a structure protein gene and a small epitope gene can also contain a promoting-agent fraction in an insertion or addition and both genes [neither of] may be destroyed.

[0021] When [of the start codon of a structure protein gene] adding a small epitope gene to the upstream immediately, in the style of [of a small epitope gene] the best, it is required, and a start codon needs to make a stop codon in the style of [of elimination and small epitope gene of the stop codon of a structure protein gene] the lowest, when [of the stop codon of a structure protein gene] adding a small epitope gene to a lower stream of a river immediately.

[0022] (Production of the 3rd recombination vector) The marker gene for making selection of a recombination virus easy is inserted by the conventional method to the 2nd recombination vector, and the 3rd recombination vector is produced.

[0023] Although especially a marker gene is not limited, a beta-galactosidase gene (Molecular And Cellular Biology, 5, 3403 (1985)), Ecogpt gene (J. Virol., 62, 1849-1854 (1988)), etc. are illustrated, for example. A marker gene is used in the status that it tied to the promoting agent's lower stream of a river.

[0024] As promoting agent who ties to a marker gene As what it will not be limited especially if it functions within the virus to incorporate, for example, is discovered within a vaccinia virus The promoting agent of the vaccinia virus gene which carries out the code of the 7.5K polypeptide The promoting agent of the vaccinia virus gene which carries out the code of (it is hereafter called 7.5K promoting agent) and the 19K polypeptide As what the promoting agent of the vaccinia virus gene which carries out the code of (it is hereafter called 19K promoting agent) and the 11K polypeptide etc. is illustrated, and is discovered within a baculovirus The promoting agent of the baculovirus gene which carries out the code of the ***** drine compounds of a baculovirus, As what the promoting agent of the baculovirus gene which carries out the code of the 10K polypeptide of a baculovirus etc. is illustrated, and is discovered within a loon poxvirus The promoting agent of the loon poxvirus gene which carries out the code of the thymidine kinase of a loon poxvirus, 7.5K promoting agent, 19K promoting agent, etc. are illustrated.

[0025] the marker gene tied to the promoting agent's lower stream of a river is boiled near [where the 2nd small epitope gene was inserted or added] the vaccinia virus configuration protein gene, it is inserted, and the 3rd recombination vector is produced The system of the easy Escherichia coli of a gene manipulation should just be used for construction this ***** of the these [1st], the 2nd, and 3rd recombination vectors.

[0026] (A construction of a recombination virus, and proteinic manufacture) Introduce into the cell which infected beforehand with the virus the 3rd recombination vector obtained by the above-mentioned technique, homology recombination is made to cause between vector DNA and the gene of a viral genome, and a recombination virus is built. If in charge of a construction of a recombination virus, that what is necessary is just to carry out according to a conventional method, the 3rd recombination vector is made to introduce in a virus by the calcium phosphate coprecipitation method, the liposome method, the microinjection, the electroporation method, etc., and the virus ends containing the recombination virus obtained is cultivated on a suitable culture medium.

[0027] A plaque is made to form by the technique suitable for the marker gene inserted by the 3rd recombination vector, and the candidate stock of the target recombination vaccinia virus is obtained. What is necessary is to carry out multistory [of the MEM containing the halogenation indolyl-beta-D-galactosidase (henceforth a ***** gal), and the agar], and just to consider as the candidate stock of the recombination vaccinia virus aiming at the plaque dyed blue in a night as a marker gene, after making a plaque form, when a beta-galactosidase gene is used.

[0028] The technique of choosing the recombination virus made into the purpose from these candidate stocks should just carry out the ***** assay using the antiserum or monoclonal antibody to the small epitope which purifies a plaque using the hybridization method which uses as a probe the small epitope gene made into the purpose, or is made into the purpose. Thus, hybrid protein will be produced, if the obtained recombination virus is infected with a host cell and cultivated.

[0029] The cell used here will not be limited especially if the virus which serves as a recipient is infected. TK-143 when the virus which serves as a recipient is a vaccinia virus (Homo-sapiens osteosarcoma origin), floor line (Homo-sapiens amnion origin), Hela (Homo-sapiens uterine cervix cancer origin), KB (Homo-sapiens nose throat cancer origin), valve flow coefficient-1 (ape kidney origin), BSC-1 (ape kidney origin), RK13 (lagomorph kidney origin), L929 (mouse connective-tissue origin), CE (****), CEF (hen fetus fibroblast), etc. are illustrated. When using a baculovirus, Sf9 cell of the Spodoptera full ***** (Spodoptera frugiperda) origin etc. is illustrated. When using a loon poxvirus, CE (****), CEF (hen fetus fibroblast), etc. are illustrated.

[0030] Thus, what is necessary is to collect and crush a cell and just to collect it, since the produced hybrid protein usually exists in large quantities in a host cell while the host cell is valid. Hybrid protein can be refined by adopting, combining suitably the fractionation method by a well-known conventional method, for example, a salting-out, a gel filtration, the ion exchange and the separation method by the affinity column chromatography, the high performance chromatography, and electrophoresis etc.

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EXAMPLE

[Example] An example is raised to below and this invention is explained to it still concretely.

(Example 1) First, manufacture of the production (refer to drawing 1) vaccinia virus genomic DNA of vaccinia virus main outer-membrane antigen p37 gene and the 1st recombination plasmid pHP37 by the cloning of the circumference cultivated the vaccinia virus LC16mO stock (it is called a vaccinia virus mO stock clinical, a virus, 3(3)13-19 (1975), and the following) by the conventional method, and was performed by the technique of J.Virol.Methods and 2,175 to 179 (1981) publication.

[0033] This genomic DNA was cut by restriction enzyme PstI and restriction enzyme HindIII, and the DNA fragments of 4452bps containing p37 gene were collected. pUC18 was cut by restriction enzyme PstI and restriction enzyme HindIII, the DNA fragment obtained previously was inserted and the first recombination plasmid pHP37 was produced. authentication of pHP37 checks the restriction enzyme site based on the base sequence of Virol., 179, and 247-266 (1990) publication -- it carried out

[0034] (Example 2) Production of the plasmid pGH37 containing the second half fraction of production (1) p37 gene of the second recombination plasmid which has synthesis of HGP-30 gene, p37 gene, and HGP-30 gene (refer to the drawing 2)

pHP37 obtained in the example 1 of reference was cut by restriction enzyme HpaI and restriction enzyme BglII, and the DNA fragments of second half partial abbreviation 1260bp of p37 gene were collected. After having carried out KI now processing of this fragment and considering as a flush end, it inserted in pBR322 ((Cold Spring Harbor Symposium, 77 [43 and 77], 1979)) which carried out full digestion by restriction enzyme DraI, and the recombination plasmid pGH37 was obtained. pGH37 merely has one DraI site in the position which lapped with the stop codon of p37 gene.

[0035] (2) Production of plasmid pGH37HGP by insertion of the synthetic DNA equivalent to HGP-30 gene to pGH37 (refer to the drawing 2)

DNA (array number 1) which carries out the code of the HGP-30 gene was compounded using the DNA-synthesis machine. This synthetic DNA has HindIII site in a center section. pGH37 gene obtained above (1) was processed by restriction enzyme DraI, the synthetic DNA which carries out the code of the HGP-30 obtained previously was inserted, and recombination plasmid pGH37HGP was obtained. Process pGH37HGP by restriction enzyme SspI, and the DNA fragment near between p37 gene and HGP-30 genes is obtained. Since pGH37HGP was processed by restriction enzyme DraI when the base sequence of this fragment was checked by *****'s et al. technique (Nucl.Acids.Res., 9,309-321 (1981)) The stop codon disappeared between HGP-30 genes in p37 gene and its lower stream of a river, and it turns out that it became the codon which carries out the code of the thyrosin.

[0036] (3) p37 gene back circumference array and production of the recombination plasmid pKPHGP30 according to addition of an array the first half of p37 gene (drawing 3, four references)

pGH37HGP obtained above (2) was cut by restriction enzyme HindIII and restriction enzyme SspI, and the DNA fragments of 361bps containing the second half fraction of HGP-30 gene were collected. Moreover, pHP37 obtained above (1) was cut by restriction enzyme PstI and restriction enzyme SspI, and the DNA fragments of about 1250 bps containing the back array of p37 gene were collected. Next, pUC18 was processed by restriction enzyme HindIII and restriction enzyme PstI, the DNA fragment of collected 361bps and the DNA fragment of about 1250 bps were inserted, and recombination plasmid pHPRear was produced.

[0037] pGH37HGP obtained above (2) was similarly cut by restriction enzyme HindIII and restriction enzyme SspI, and the DNA fragments of 91bps containing a part for the first portion of HGP-30 gene were collected. Moreover, pHP37 obtained above (1) was cut by restriction enzyme KpnI and restriction enzyme SspI, and the DNA fragments of about 1050 bps containing the front array of p37 gene were collected. Next, pUC18 was processed by restriction enzyme HindIII and restriction enzyme KpnI, the DNA fragment of collected 91bps and the DNA fragment of about 1050 bps were inserted, and recombination plasmid pHKFront was produced.

[0038] Thus, the obtained DNA fragments of about 1510 bps which rearrange, cut plasmid pHPRear by restriction enzyme PstI and restriction enzyme HindIII, and include the array of the second half fraction of HGP-30 gene and the back circumference fraction of p37 gene were collected. Moreover, recombination plasmid pHKFront was cut by restriction enzyme PstI and restriction enzyme HindIII, and the DNA fragments of about 1140 bps containing the array of a part for the first portion of HGP-30 gene and the front fraction of p37 gene were collected. pUC18 was processed by restriction enzyme PstI and restriction enzyme KpnI, two collected fragments were inserted and the recombination plasmid pKPHGP30 was obtained.

[0039] (4) Production of the 2nd recombination plasmid pPHGP30 by addition of p37 gene front array (refer to the drawing 5)

The DNA fragments of about 2650 bps which rearrange, cut a plasmid pKPHGP30 by restriction enzyme PstI and restriction enzyme KpnI, and contain p37 gene back circumference partial array and p37 gene obtained above (3) were collected.

[0040] Moreover, the DNA fragments of about 1890 bps which rearrange, cut a plasmid pHP37 by restriction enzyme KpnI and restriction enzyme HindIII, and include the front circumference partial array of p37 gene obtained above (1) were collected. pUC18 was processed by restriction enzyme PstI and restriction enzyme HindIII, two collected fragments were inserted and the recombination plasmid pPHGP30 was produced.

[0041] (Example 3) Production of 3rd recombination plasmid pPHGP30Z by inclusion of the beta-galactosidase gene to the 2nd recombination plasmid pPHGP30 (refer to the drawing 6)

(1) The production beta-galactosidase gene of pNZ76 which the beta-galactosidase gene to a vaccinia virus 7.5K promoting-agent lower stream of a river incorporated cut and collected the plasmids pMA001 of Gene and 28,127 to 132 (1984) publication by restriction enzyme BamHI. The plasmid pAK8 (JP,64-74982,A) which connected the polylinker with 5K promoting agent's lower stream of a river was processed by BamHI, the beta-galactosidase gene collected previously was inserted and the recombination plasmid pNZ76 was obtained.

[0042] (2) pNZ76 obtained by the production above (1) of 3rd recombination plasmid pPHGHP30Z by inclusion of the beta-galactosidase gene to the 2nd recombination plasmid pPHGHP30 was cut by restriction enzyme HindIII and restriction enzyme SmaI, the DNA fragments with which 7.5K promoting agent and the beta-galactosidase gene were connected were collected, KI now processing was carried out further, and the DNA fragment of about 4 Kbps was obtained. The DNA fragment of about 4 Kbps which processed the plasmid pPHGHP30 by restriction enzyme SmaI by rearranging, and were obtained previously obtained in the above-mentioned example 2 of reference (4) was inserted, and recombination plasmid pPHGHP30Z was produced.

[0043]

(Example 4) A vaccinia virus WR stock is inoculated into RK-13 cell cultivated by the culture bottle of 2.25cm of production of a recombination vaccinia virus at a rate of 0.1 p.f.u. / cell. Recombination plasmid pPHGHP30Z obtained in the example 3 of 10 microg is melted in a 2.2ml sterilized water 45 minutes after. By Hidaka's et al. (protein, a nucleic acid and an enzyme, 27,340- (1985)) technique, DNA-calcium phosphate coprecipitate was built and the 0.5ml was dropped at infection RK-13 cell. It put on 37 degrees C and 7% CO2 incubator gently for 30 minutes, and 4.5ml of the MEM which contains fetal calf serum 5% was added. Culture medium was exchanged 3 hours [the] after, the 3 times freeze thawing was cultivated and carried out for every cultured cell in 37 degrees C and 7% CO2 incubator, and the virus liquid containing the recombination field was obtained for 48 hours.

[0044] The above-mentioned virus liquid is inoculated into RK-13 cell cultivated by 10cm Petri dish for selection of the recombination field. The laminating of 0.8% agarose and the MEM which contains fetal calf serum 5% is carried out 30 minutes after. The laminating of the MEM which contains agarose and a 0.5mg [/ml] ***** gal (product made from BRL) in an infected cell 0.8% will be carried out after incubation for three days. In order that a virus may be sampled by the Pasteur pipette from the plaque which dyed blue 14 hours after, this may be suspended in the phosphate buffered saline (henceforth PBS) which contains gelatin 2% and a part may carry out a dot hybridization. The spot was carried out to nylon or the nitrocellulose membrane, and the remainder was saved at -20 degrees C.

[0045] the membrane which carried out the spot processed processing for 5 minutes for 5 minutes the back with 1M tris hydrochloric-acid buffer solution for 10 minutes in 0.5N sodium-hydroxide aqueous solution with 1.5M sodium chloride and 0.5M tris hydrochloric-acid buffer solution. You made it saturated with 2 double SSC (for SSC to contain 0.15M sodium chloride and 0.015M sodium citrate 1 time), and 80 degrees C was printed for 2 hours.

[0046] Then, this was processed 68 degrees C for 2 hours with the mixed liquor which consists of 4 times SET (0.6M sodium chloride, 0.08M tris hydrochloric-acid, EDTA [of 4mM], pH 7.8)-10 times Denhardt-0.1% SDS. By 4 times SET-10 times Denhardt seven to 50 microg [/ml] O [-0.1%SDS-0.1%Na4P2] denaturation salmon sperm DNA, and ***** by 32P, HGP-30 synthetic gene which carried out the indicator was put in, and the hybridization of the 68 degrees C was carried out for 14 hours. The membrane and the X-ray film were piled up after washing, autoradiography was performed, and the spot in which a film carries out a melanism was chosen.

[0047] The virus liquid (amount m.o.i.=of viruses 3×10^{-6}) corresponding to the spot which carried out the melanism is again inoculated into RK-13 cell. The laminating of 0.8% agarose and the MEM which contains fetal calf serum 5% is carried out 30 minutes after. The laminating of the MEM which contains agarose and a 0.5mg [/ml] ***** gal 0.8 more% will be carried out after incubation for three days. Purification was repeated until it performed the same operation as the above and all the appearing plaques carried out the melanism by the dot hybridization about the plaque which dyed blue 14 hours after. In this way, the obtained virus is the target recombination vaccinia virus, and named this v37HGP30.

[0048] (Example 1 of a comparison) Production of the recombination plasmid which does not have structure protein p37 gene of the construction (1) virus of a recombination vaccinia virus which does not have p37 gene but has small epitope HGP-30 gene, but has small epitope HGP-30 gene (refer to the drawing 3)

pAK8 was processed by restriction enzyme SalI and restriction enzyme EcoRI, HGP-30 synthetic gene with the base sequence (it has SalI site in array number 2 and 5' upstream side, and has EcoRI site in 3' lower-stream-of-a-river side) compounded with the DNA-synthesis machine was inserted, and recombination plasmid pAK8HGP30 was produced. In pAK8HGP30, a vaccinia virus TK gene is destroyed and it is in the status that HGP30 synthetic gene which has a start codon and a stop codon forward and backward on vaccinia virus 7.5K promoting agent's lower stream of a river was combined.

[0049] (2) as a construction recombination plasmid of a recombination vaccinia virus, instead of pPHGHP30Z, it rearranges and plasmid pAK8HGP30 is used, except the thing which obtained above (1) and for which a virus is chosen with 5-*****-2'-deoxyuridine, it rearranges by the same technique as an example 4, a vaccinia virus is built, and it was obtained — it rearranged and the vaccinia virus was named vTKHGP30

[0050] (Example 5) vTKHGP30 built in recombination vaccinia virus v37HGP30 or the example of a comparison of this invention of m.o.i.=1 was inoculated, respectively, virus liquid was removed into RK-13 cell proliferated by the MEM which is in chamber sliding for manifestation tissue culture by the infected cell of a recombination vaccinia virus, and contains fetal calf serum 5% after 1 hour neglect at 37 degrees C, respectively, the cell was washed into it by MEM, and the MEM which contains fetal calf serum 5% was 16 hours after, except for MEM, it washed by PBS lightly, acetone processing was carried out for 5 minutes at the room temperature after air-drying, and the cell was fixed, respectively.

[0051] The specific issue by fluorescence was observed using the fluorescence microscope by the indirect fluorescent antibody technique which uses anti-HGP-30 monoclonal antibody (made in ***** Technologies, Inc.) as a primary antibody, and uses an iso ***** acid fluorescein joint anti-mouse IgG antibody (product made from TAGO) as a second antibody. Consequently, although recombination vaccinia virus v37HGP30 of this invention had discovered a lot of small epitope HGP-30, it rearranged, and the vaccinia virus vTKHGP30 completely discovered small epitope HGP-30, and it turns out [****] that it does not have the structure protein gene of the example of a comparison.

[0052] (Example 6) Int RK-13 cell proliferated using the MEM using the Western blotting which rearranges and contains fetal calf serum in the incubation plate of 2.5% 10cm of authentication of a manifestation of the hybrid

protein in a vaccinia virus infected cell membrane. Recombination vaccinia virus v37HGP30 or the vaccinia virus WRormo stock built in the example 4 of o.i.=1 is inoculated, respectively. Virus liquid was removed after 1 hour incubation at 37 degrees C, respectively, the cell was washed by MEM, and the MEM which contains fetal calf serum 5% was added. 16 hours after, except for MEM, it was washed by PBS lightly, it moved to the centrifuging tube, the at-long-intervals core separation was carried out by 3000rpm for 5 minutes, and cells were collected.

[0053] Next, after having added the ***** buffer (Natura, 227, 680 (1970)) of 100microl and suspending a cell again, it processed for 5 minutes at 100 degrees C. Next, the supernatant liquid obtained by the centrifugal separation for 10 minutes by 15,000rpm was made into the sample, and the Western blotting was performed according to the conventional method. In addition, the used primary antibody is the same as that of what was used in the example 5. As for the vaccinia virus used as a parent strain, by v37HGP30 infected cell, a band was not accepted at all to the band with which about 40 Kds clarified having been accepted as a result of the Western blotting. It turns out that the hybrid protein with which HGP-30 protein was added to p37 protein in v37HGP30 infected cell is discovered with this.

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DESCRIPTION OF DRAWINGS

[Brief Description of the Drawings]

[Drawing 1] It is explanatory drawing having shown the production technique of the recombination plasmid pHP37.

[Drawing 2] It is explanatory drawing having shown the production technique of recombination plasmid pGH37HGP.

[Drawing 3] It is explanatory drawing in which having rearranged with recombination plasmid pHPrear and having shown the production technique of plasmid pHKFront.

[Drawing 4] It is explanatory drawing having shown the production technique of the recombination plasmid pKPHGP30.

[Drawing 5] It is explanatory drawing having shown the production technique of the recombination plasmid pPHGP30.

[Drawing 6] It is explanatory drawing having shown the production technique of recombination plasmid pHPGHP30Z.

[0054]

[Layout Table] Array Number: One

Length:94 of an array

The mold:nucleic acid of an array

The number:double strand of a chain

Topology: The shape of a straight chain

The nucleic acid besides modality: of an array Synthetic DNA

Array

ATAGCGTAC ATCAAAGGAT AGATGTAAAA GACACCAAGG AAGCTTTAGA GAAGATAGAG 60

GAAGAGCAA AACAAAAGTA AGAAAAAGGC TTAA 94

[0055]

[Layout Table] Array Number: Two

Length:104 of an array

The mold:nucleic acid of an array

The number:double strand of a chain

Topology: The shape of a straight chain

The nucleic acid besides modality: of an array Synthetic DNA

Array

TCGACATGT ATAGCGTACA TCAAAGGATA GATGTAAAAG ACACCAAGGA AGCTTTAGAG 60

AAGATAGAG GAAGAGCAAA ACAAAGTAA GAAAAAGGCT TAAG 104

[Translation done.]